PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF SELECTED WILD EDIBLE AND CULTIVATED MUSHROOMS OF NEPAL

A Dissertation

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DECLARATION

The research work entitled " **Phytochemical screening and antioxidant activity of selected wild edible and cultivated mushrooms of Nepal**" submitted by me at the Institute of Science and Technology, Tribhuvan University for the partial fulfillment of master's degree in Botany is work carried out under the supervision of **Assoc. Prof. Dr. Hari Prasad Aryal**. The research work shown here has not been submitted for the granting of any degree.

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LETTER OF APPROVAL

The M.Sc. Dissertation entitled **"Phytochemiccal screening and antioxidant activity of selected wild edible and cultivated mushrooms of Nepal"** submitted by **Ms. Madhu Thapa Magar** has been accepted for the partial fulfilment of Master's Degree in Botany (Plant Pathology and Applied Mycology Unit).

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Madhu Thapa Magar

ABSTRACT

Wild edible mushrooms have been widely used as nutrient-rich food since ancient times, and some of them are also produced on an industrial scale. In this study, the three most common cultivated mushrooms (*Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus*) and three wild edible mushrooms (*Scleroderma cepa*, *Termitomyces microcarpus* and *Termitomyces schimperi*) were selected for the study. The objectives of the study were to analyze and compare the bioactive compounds present and antioxidant activity of wild edible and commonly cultivated mushrooms. The Total Phenolic Content value was estimated by using the Folin-Ciocalteu method. To determine the antioxidant potential of the samples, DPPH (2, 2-Diphenyl-1-picrylhydrazyl) and ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) scavenging assays were employed. From the preliminary phytochemical screening, it was observed that both wild and cultivated mushrooms tend to contain bioactive compounds such as Alkaloids, Steroids, Terpenoids, Polyphenols, Cardiac glycosides, and Saponins. Methanolic extracts of all three wild mushrooms exhibited higher TPC than the cultivated mushrooms. Highest TPC was found in water extract of *S. cepa* with the value of 36.57±0.81 mg GAE/ g dry extract. Lowest TPC was found in methanol extract of cultivated mushroom, *A. bisporus* with the value of 11.60±0.08 mg GAE/ g dry extract. The antioxidant assay revealed that all three wild mushrooms are strong inhibitors of free radicals like DPPH with the lowest IC_{50} values than the cultivated mushrooms. But in the case of ABTS assay, only *S. cepa* and *T. microcarpus* had a lowest IC_{50} values than other mushroom samples. Lowest IC_{50} value in inhibiting DPPH radical was observed in methanolic extract of *S. cepa* with a value of 0.744 mg/mL. And, lowest IC_{50} value in inhibiting ABTS radical was also found in water extract of *S. cepa* with the value of 0.583 mg/mL. This study revealed that there was a correlation between the TPC and ABTS radical scavenging potential of the samples. But no significant correlation could observed in the DPPH radical scavenging assay. Further study should focus on exploring many other edible mushrooms and identifying specific antioxidant compound present in them. But, initial focus of the study should focus on the toxicity of wild mushrooms because many mushrooms in the wild could be poisonous to human kind.

Keywords: Bioactive compounds, total phenolic content, *S. cepa*, *T. microcarpus*, *T. schimperi*

शोधसार

जंगली खान योग्य च्याउहरु धेरै समुदायहरुमा महत्वपुर्ण पोषण तत्वको रुपमा लिइन्छ र पुरातन समयदेखि दैनिक आहारको रुपमा प्रयोग हुदै आएको छ । यस अध्ययनमा तीनओटा सामान्यतया खेति गरिएका च्याउहरु (*Agaricus bisporus, Lentinula edodes* र *Pleurotus ostreatus*) र तीनओटा धेरै प्रयोग गरिएका जंगली च्याउहरु (*Scleroderma cepa*, *Termitomyces microcarpus* र *Termitomyces schimperi*) लाई अध्ययनको लागि चयन गरिएको थियो । यस अध्ययनको उद्देश्य जंगली खान योग्य र सामान्यतया खेति गरिएका च्याउहरुको Phytochemistry र Antioxidant गतिविधि को विश्लेषण र तुलना गर्नु थियो । Folin- Ciocalteu विधि प्रयोग गरेर Total Phenolic Content को मात्रा अनुमान गरियो । च्याउका नमुनाहरुको antioxidant क्षमता निर्धारण गर्न DPPH (2, 2-Diphenyl-1-picrylhydrazyl) र ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) Scavenging गतिविधिहरुको मुल्याङ्कन गरियो । प्रारम्भिक फाइटोकेमिकल स्किनिङबाट यो देखियो कि जंगलि र खेति गरिएका च्याउहरुमा Alkaloids, Steroids, Terpenoids, Polyphenols, Cardiac glycosides, र Saponins जस्ता तत्वहरु पाइन्छन् । तीनओटै जंगली च्याउहरुको मिथानोलिक extracts ले खेति गरिएका च्याउहरुको तुलनामा TPC उच्च देखाएको थियो । उच्चतम TPC, *S. cepa* को water extract मा पाइयो जसको मात्रा 36.57±0.81 mg GAE/ g dry extract थियो । सबैभन्दा कम TPC खेति गरिएको च्याउ *A. bisporus* मा 11.60±0.08 mg GAE/ g dry extract को मात्रामा पाइयो । तीनओटै जंगलि च्याउहरुले खेति गरिएको च्याउहरुको तुलनामा सबैभन्दा कम IC_{50} मान ल्याएर DPPH फ्री रेडिकलको सबैभन्दा बलियो अवरोधहरुको रुपमा प्रमाणित भएको छ । तर ABTS फ्री रेडिकलको सन्दर्भमा, *S. cepa* र *T. microcarpus* ले मात्र अन्य च्याउका नमुनाहरुको तुलनामा कम IC_{50} मान ल्याएको छ । 0.744 mg/mL IC_{50} मानको साथ *S. cepa* को मिथानोलिक extract, DPPH रेडिकललाई अवरोध गर्ने सबैभन्दा बलियो नमुनाको रुपमा पाइएको छ । ABTS रेडिकलको सन्दर्भमा पनि, सबैभन्दा कम IC_{50} मान, *S. cepa* मा 0.583 mg/mL को मात्रामा पाइयो । यस अध्ययनले च्याउका नमुनाहरुको TPC र ABTS रेडिकललाई रोक्ने क्षमताको बीचमा सहसम्बन्ध रहेको देखाएको छ । तर TPC र DPPH रेडिकललाई रोक्ने क्षमताको बीचमा कुनै महत्वपुर्ण सम्बन्ध देखाएन । थप अध्ययनले धेरै अन्य खाद्य च्याउहरु अन्वेषण गर्न र तिनिहरुमा अवस्थित विशिष्ट एन्टिअक्सिडेन्ट पदार्थहरु पहिचान गर्नमा ध्यान केन्द्रित गर्नुपर्दछ । तर, अध्ययनको प्रारक्भिक फोकस जंगली च्याउको विषाक्ततामा केन्द्रित गर्नुपदर्छ किनभने जंगलमा धेरै च्याउहरु मानव जातीको उपयोगका लागी विषाक्त हुन सक्दछ ।

शब्दकुञ्जी: जैविक सक्रिय यौगिक, Total phenolic content, *S. cepa*, *T. microcarpus*, *T. schimperi*

LIST OF ACRONYMS AND ABBREVIATIONS

µg	Microgram
ABTS	2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
Cm	Centimeter
Conc.	Concentration
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
GAE	Gallic Acid Equivalent
GA	Gallic acid
g	Gram
IC_{50}	Half-maximal inhibitory concentration
Inhi.	Inhibition
mg	Milligram
mL	Milliliter
NAST	Nepal Academy of Science and Technology
SE	Standard Error
TPC	Total Phenolic Content

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1. INTRODUCTION

1.1 General background

Mushrooms, also known as Macrofungi, are a group of species belonging to the phyla Basidiomycota and Ascomycota having distinctive fruiting bodies that can be hypogenous or epigenous. Fungi or mushrooms are heterotrophic plants that do not contain chlorophyll; to meet their daily demands, they consume premade food components from other species.

Mushrooms play a significant role in associations with mycorrhizae, saprotrophs, parasites, and insects in various ecosystems (Paloi et al., 2023). Globally, there are an estimated 53,000 to 110,000 mushroom species. Approximately 14,000 species have been officially described to date (Mueller et al., 2007).

1.1.1 Diversity of mushrooms in Nepal

Nepal is a landlocked Himalayan country with considerable elevation differences and a diverse range of climates that has been named a natural biodiversity hotspot. With five climate zones within 150 kilometers of each other, one can swiftly go from a conventional tropic to a constantly cold Arctic-like zone (Hai Bang et al., 2014). This diversity has provided the country with a diverse phytogeography as well as economically lucrative Mycoflora. The wild mushrooms of Nepal are categorized into 108 families, 357 genera, and 1291 species, with 34 endemic species, 159 edible mushrooms, 74 medicinal mushrooms, and 100 toxic mushrooms (Devkota and Aryal, 2020). Wild edible mushrooms are gathered as a vital food resource (Devkota, 2008), and they provide a significant source of income for rural communities (Adhikari, 2000, Christensen & Larsen 2005, Devkota, 2008). Mushrooms are regarded as an essential and prestigious component of culture in the upper highlands of Nepal (Christensen et al., 2008).

1.1.2 Nutritional value of mushrooms

Mushrooms are vital nutritional components in many cultures and have been used as a supplement to daily diets since ancient times. Even before thorough research and validation of their capabilities, mushrooms have been used as food and medicine for many centuries. Mushrooms are considered a nutritious food since they contain carbs, fiber, protein, essential amino acids, unsaturated fatty acids, vitamins, low calories, and minerals such as potassium, iron, copper, zinc, and manganese (Sanchez, 2004). Besides this, mushrooms are also a rich source of vitamins B_1, B_2, B_{12}, C, D, and E (Mattila et al., 2001, Heleno et al., 2010). Mushrooms have a low calorie count, are fat-free, cholesterol-free, gluten-free, and contain very little sodium (Assemie and Abaya, 2022). Apart from being a nutrient-rich food source and non-toxic medications with physiological benefits, mushrooms include a variety of secondary metabolite chemicals that contribute to a variety of biological activities (Mirfat et al., 2010).

1.1.3 Secondary metabolites in edible mushrooms

Secondary metabolites do not directly participate in an organism's fundamental life activities (growth, development, and reproduction), but they frequently play critical roles in adaptation, defense, and interactions with the environment. These molecules are more varied and generated in lesser quantities than primary metabolites. Mushrooms include many beneficial phytochemicals such as phenolics, tocopherols (Vitamin E), ascorbic acid, and carotenoids, which have been shown to have noteworthy antioxidant and antibacterial activities (Barros et al., 2008). They also mentioned that Phenols were the most abundant antioxidant component discovered in mushroom extracts.

A vast range of chemicals with various biogenetic origins have been identified from the edible mushrooms (Jacinto-Azevedo et al., 2021). Three edible mushrooms, *Boletus edulis*, *Lentinus edodes*, and *Xerocomus badius*, were shown to contain α-, β-, and γ-tocopherols (Heleno et al., 2015). Cultivated mushrooms, such as *Boletus* spp., *Agrocybe aegerita*, and *Pleurotus eryngii*, were found to contain fiber, chitin, beta-glucan, and total phenols in both raw and cooked samples (Manzi et al., 2004). Secondary metabolites were discovered in various edible mushrooms, with total phenolics being the most abundant, followed by flavonoids and ascorbic acid (Ramesh and Patter, 2010). *Agaricus bisporus* was assessed for its antioxidant activity both *in vivo* and *in vitro*, and it was discovered to be more effective against superoxide radicals, hydroxyl radicals, and ferric ion reduction (Ferreira et al., 2007). *Inonotus obliquus*, a well-known medicinal mushroom, was discovered to contain three types of inobilins and three types of phelligridins that had a high DPPH and ABTS radical scavenging effect (Lee et al., 2007). Mushrooms include beta-carotene, and linoleic acid which induce inhibition of auto-oxidation (Karacsonyi and Kuniak, 1994). Mushrooms were found to contain ergosterol, which is a precursor for the production of vitamin D and is known for its efficient involvement in antioxidant activity (Zeb and Lee, 2021). Tocopherols have also been discovered in mushrooms and are a powerful scavenger of free radicals, therefore they contribute to protecting the heart (Kany et al., 2019).

Secondary metabolites found in mushrooms include those with anti-inflammatory, anti-cancer, anti-bacterial, antiviral, and anti-viral properties (Muszynskaa et al., 2018). The medicinal mushrooms including *Polyporus umbellatus* and *Polyporus alveolaris* contain different types of polypeptides and cytotoxic steroids were discovered to have many therapeutic properties (Jiang and Sliva, 2010). The presence of certain phenolic compounds, such as phenolic acids, hydroxycinnamic acids, stilbenes, and oxidized polyphenols, has been linked to the inflammatory effects of several mushroomsm(Cote et al., 2010)

1.1.4 Free radicals

Free radicals are defined as any molecular species that may exist independently and has an unpaired electron in an atomic orbital. Many radicals are highly reactive and inherently unstable. They can act as oxidants or reductants by either giving or receiving an electron from other molecules (Cheeseman and Slater, 1993). These chemicals are produced either internally by regular cell metabolism or outside by sources like pollution, tobacco smoke, radiation, and medications (Bagchi and Puri, 1998).

Ironically, oxygen, an element necessary for life, can harm the human body in some circumstances. Free radicals are created as a result of mitochondrial ATP (adenosine triphosphate) synthesis when cells use oxygen to generate energy. These byproducts frequently include reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the cellular redox process (Pham-Huy et al., 2008). ROS and RNS are acronyms for free radicals and various non-radical reactive derivatives, also known as oxidants. Free radicals have various useful effects on the organism when kept at low or moderate quantities. They are required, for example, to manufacture some cellular structures and to be employed by the host defence system to combat infections (Pizzino et al., 2017). However, in excess, free radicals and oxidants cause oxidative stress, which is a destructive process that can negatively affect various cellular components. They can interact with other biological components in the body, such as protein, DNA, and lipids, and have been linked to chronic and degenerative diseases such as cancer, autoimmune disorders, aging, cataract rheumatoid arthritis, cardiovascular disease, and neurodegenerative disease (Lobo et al., 2010). All inflammatory disorders, ischemic diseases, hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension, preeclampsia, neurological disorders, alcoholism, smoking-related diseases, and many other conditions are thought to be significantly impacted by oxidative stress (Stefanis et al., 1997).

1.1.5 Antioxidants

Antioxidants are substances that reduce or eliminate the effects of free radicals. Antioxidants may protect cells in many ways that they can prevent ROS-induced auto-oxidative chain reaction, lower localized oxygen concentrations, and convert ROS to non-radical species (Oroian, 2015). A healthy balance of free radicals and antioxidants is required for appropriate physiological function.

Synthetic antioxidants have largely replaced natural antioxidants, owing to their superior stability and performance, low cost, and widespread availability. Despite the widespread usage of synthetic antioxidants, questions regarding their safety have surfaced over time. Long-term use of synthetic antioxidants has been linked to a number of health issues, including gastrointestinal issues, skin allergies, and, in certain cases, an elevated risk of cancer (Lourenço et al., 2019). Because of their possibility as carsinogens, synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have aroused concern about them (Rahman et al., 2008). As a result, there is a lot of interest in acquiring and using antioxidants from natural sources because they are thought to be safe. As a result, there has been a rise in the search for effective, non-toxic natural compounds with antioxidant properties in recent years.

1.2 Justification of the study

Knowing the adverse effects of synthetic antioxidants has led to an increase in research for naturally occurring antioxidants. Cultivated mushrooms are widely accepted by consumers in more countries (Elmastas et al., 2007). Despite this, wild mushrooms are playing a important role in human diets because of their pharmacological and nutritional qualities. Many studies have been conducted on wild and cultivated mushrooms in Nepal, however little is known about the antioxidant capabilities of both wild and produced species of mushrooms. To understand the therapeutic characteristics of commonly used mushrooms, it is necessary to determine the bioactive chemicals present and the antioxidant activity of both wild and farmed kinds. The information from this research would be a valuable reference for further study of mushrooms for their medicinal properties

1.4 Objectives of the study

The general objective of the research is to assess the bioactive compounds and examine the antioxidant activity of selected wild edible and cultivated species of mushrooms.

The specific objectives are

- To screen the secondary metabolites present in the mushroom samples
- To determine and compare the Total Phenolic Content and antioxidant activity of wild and cultivated mushrooms in different extracts.
- To investigate the correlation between Total Phenolic Content and Antioxidant activity in both wild and cultivated mushrooms.

1.5 Limitations of the Study

- This study only covers the class of secondary metabolites; it does not cover the specific compounds found in the mushroom sample.
- This study only gives information about the bioactive chemicals present and the antioxidant activity of natural compounds; other areas of work, such as domestication, media optimization, and diversity, were not performed.

2. MATERIALS AND METHODS

2.1 Study area

One of the wild edible mushroom *Scleroderma cepa* was collected from the ward-05 of Shahid Lakhan rural municipality. It is located in Gorkha district, Gandaki province of Nepal. It scatters across 149.03 square kilometers of geographical area. Main settlements in the region include Magar, and Gurung. It has a subtropical climate and the rainy season extends from June to September. Vegetation is dominated by mixed forests of *Shorea robusta* (Sal), *Castanopsis indica,* and *Schima wallichii*. Many households in the area used to collect and consume wild mushrooms for culinary purposes.

The two wild edible mushrooms *Termitomyces microcarpus* and *Termitomyces microcarpus* were collected from the ward-08 of Tansen Municipality. Tansen Municipality is located in Palpa district, Lumbini province of Nepal. The Municipality occupies an area of 109.8 square kilometers. The climate is subtropical and temperature varies from 4°C to a maximum 37°C. Vegetation is dominated by the *Shorea robusta* (Sal) forest. The area has the main settlement of the Magar community and they used to collect, sell, and consume mushrooms. The study areas are shown in Fig 1.

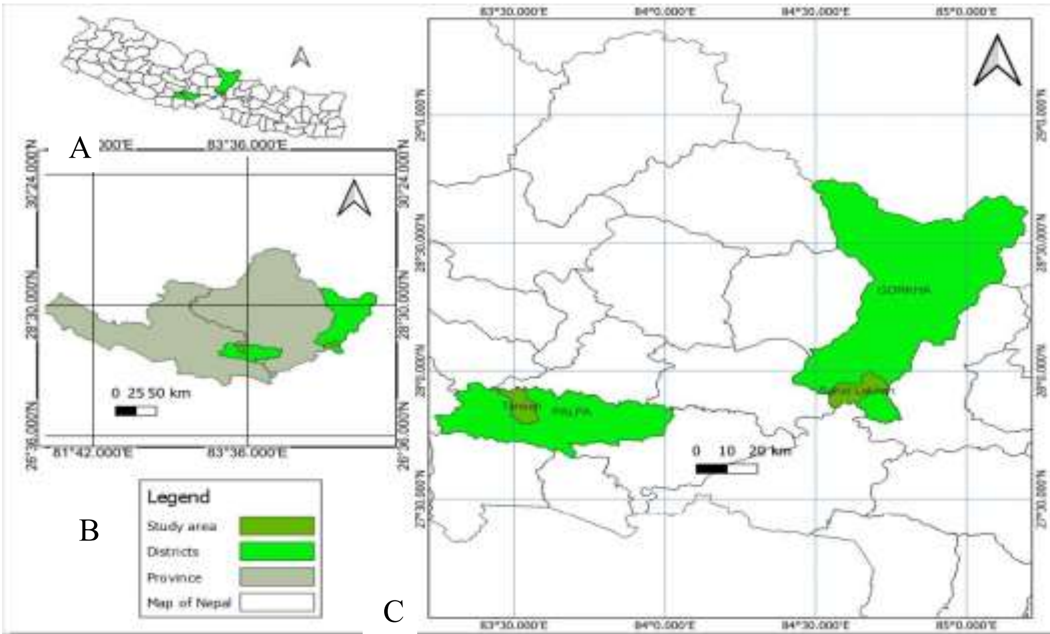


Figure 1: Study area. A. Map of Nepal showing Gorkha and Palpa districts. B. Gorkha and Palpa district. C.Shahid Lakhan Rural Municipality and Palpa Municipality

2.2 Materials

2.2.1 Mushroom sample

The entire fruiting bodies of wild edible mushrooms (*S. cepa, T. microcarpus,* and *T. schimperi*) and cultivated mushrooms (*A. bisporus, L. edodes,* and *P. ostreatus*).

2.2.1.1 Cultivated mushrooms selected for the study

Several species of mushrooms are cultivated in the world. However, a few species are commercially cultivated in Nepal. The three most common commercially cultivated mushrooms, *A. bisporus, L. edodes*, and *P. ostreatus*, were selected to examine their phytoconstituent content and antioxidant activity. They are shown in Figure 2.

Agaricus bisporus **(J.E. Lange) Imbach**

A. bisporus (white button mushroom) is the most important commercially grown fungus in the world. It was the first mushroom cultivated in Nepal (Shrestha and Dhakal, 2014). This mushroom is a popular healthy meal due to its high nutrient content, which includes carbs, proteins, lipids, fibers, minerals, and vitamins. (Atila et al., 2017).

Lentinula edodes **(Berk.) Pegler**

L. edodes (Shitake) is one of the most popular mushrooms grown on a commercial basis in Nepal (Raut, 2019). It is the world's second most popular mushroom, owing not only to its nutritional benefits but also to its potential for therapeutic applications (Bisen et al., 2010). It is an edible and medicinal mushroom with a unique fungal aroma and the ability to cure numerous diseases such as cancer, diabetes, hypotension, inflammatory, nociceptive, and hypocholesterolemic (Li et al., 2018).

Pleurotus ostreatus **(Jacq.) P. Kumm**.

P. ostreatus most commonly cultivated in Nepal. It is considered as an important food with medical properties because of its richness in nutrients, including easily available proteins, vitamins, and bioactive substance like pleuran and lovastatin (Piska et al., 2017).

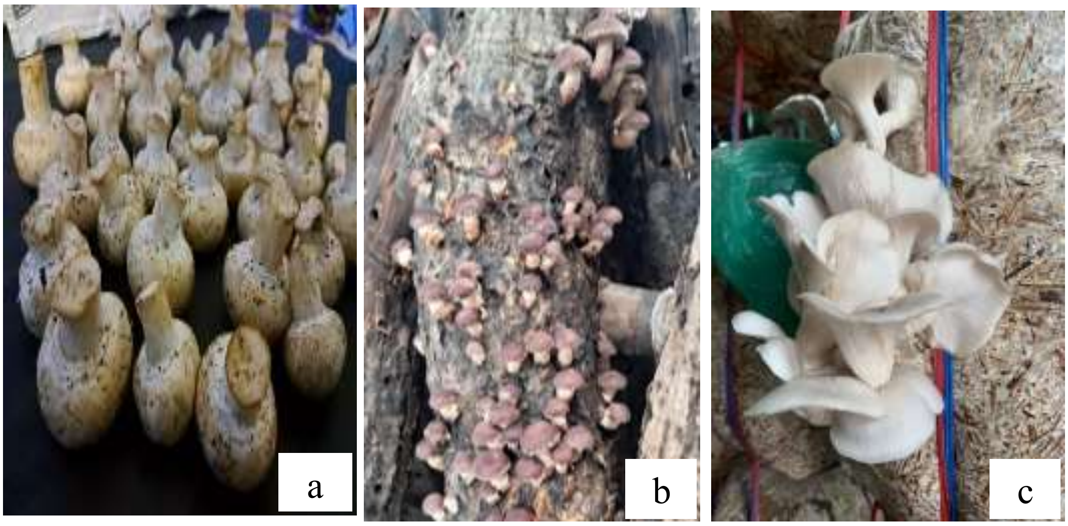


Figure 2: Cultivated mushrooms. (a) A. bisporus. (b) L. edodes. (c) P. ostreatus

2.2.1.2 Wild edible mushrooms selected for the study

Based on the most dominant and commonly used by local peoples of Nepal, three wild edible mushrooms, *Scleroderma cepa*, *Termitomyces microcarpus*, and *Termitomyces schimperi* were selected for their phytochemical analysis and antioxidant activity. They are shown in Figure 3.

***Scleroderma cepa* Pers.**

It is an ectomycorrhizal fungus belonging to the Sclerodermataceae family. It grows alone or in groups in short grass, gardens, and along pathways. The fruiting body is brownish, buried to epigeous, measuring 2-5cm in width. Peridium is thick, tough, and increasingly finely broken or areolate, particularly in the top region. Spores are expelled through uneven splitting or cratering of the apex.

***Termitomyces schimperi* (Pat.) R. Heim**

It is a fungal species in the Lycophyllaceae family that is associated with termite species. It features a large white sporocarp (12-15cm in diameter) that is initially coated in a brownish squamulose layer before, eventually becoming simply a detachable sheet at the top of the cap and below the ring. The pseudorhiza tapers towards its origin in the termite nest and can grow to be about 40-50 cm long. The sporocarps appear in clusters around the termite mound. These mushrooms grow from Shrawan to Asoj

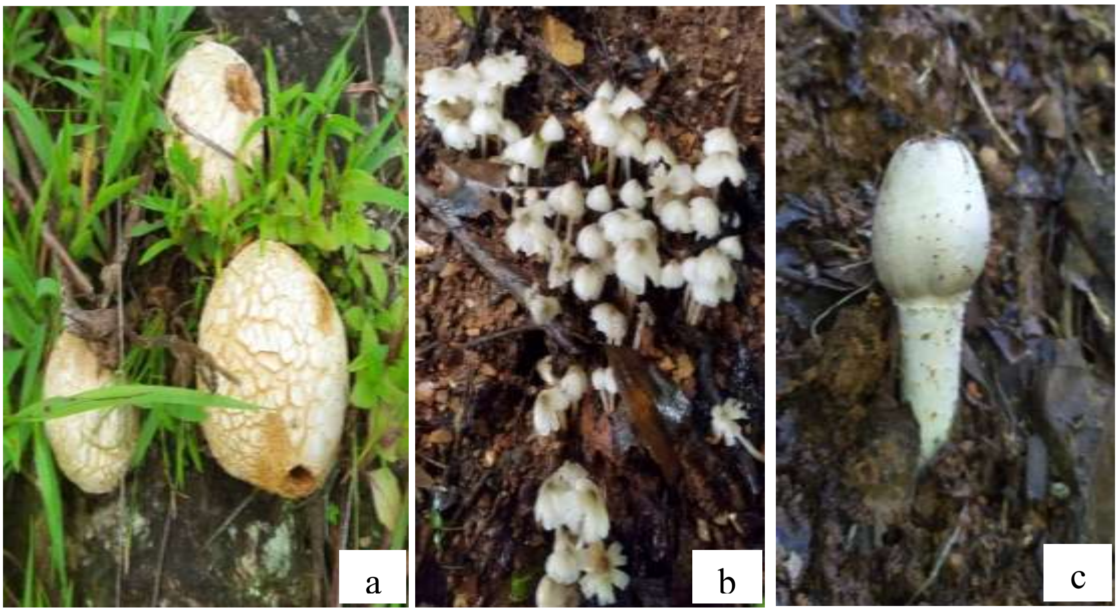


Figure 3: Wild edible mushrooms. (a) S. cepa. (b)T. microcarpus. (c) T. schimperi

(August to October). This is sold for up to Rs. 800 to 1000 per kg in the local market. It is regarded as a highly tasty and nutritious cuisine in the local community.

Termitomyces microcarpus **(Berk. & Broome) R. Heim**

It is also a fungus species belonging to the Lycophyllaceae family. It has a tiny cream white to light gray pileus with a cream dark the center. A cap is up to 1-3cm in diameter, split from its margins, and resembles flower petals. Lamellae are free, crowed and white in color, turning creamy at maturity. Stipe is up to 4-10cm long, slender, white, fleshy, smooth, and absence of annulus. Pseudorhiza is absent. This mushroom's fruiting season lasts from July to September. It is also the most popular mushroom species among the locals, and it is regarded as a delicious and nutritious food. After cooking, the mushromms become pulpy and have a meat-like flavor. It has no market sales record.

2.2.2 Chemicals

The chemicals used were Dragendroff Reagent. 2% hydrochloric acid (HCl), chloroform ($CHCL_3$), concentrated H_2SO_4, acetic acid, 10% NH_4OH, 10% HCL, 10% NH_3, 1% $FeCl_3$, Na_2CO_3, ether. A 25% ammonia solution, concentrated HCl, 1% methanolic KOH, 1% methanolic 3, 5-dinitrobenzoic acid, 2% copper sulphate solution, ethanol, methanol, Hexane, DPPH, Folin-Ciocalteu reagent (Loba Chemical Pvt. Ltd), and Gallic Acid.

2.2.3 Instruments

Soxhlet Apparatus, Water Bath, test tubes, Beakers, Volumetric Flasks, Micropipette, Elisa Microplate reader (Epoch 2, BioTek Instruments), 96-well microplates, Low-Temperature Constant Bath, Hot air oven, Desiccator, Vacuum Desiccator, Weighing machine.

2.3 Methods

2.3.1 Sample collection

The wild edible mushrooms was collected during August to October 2022. Fresh and mature mushrooms were collected from the mushroom farms. *Lentinula edodes* and *Pleurotus ostreatus* were collected from the Mushroom Seed Nepal and Research Center Pvt. Ltd., Bhaktapur. *Agaricus bisporus* was collected from Sonitpur Mushroom and Spawn house Pvt. Ltv. Thankot, Kathmandu. Collected Mushrooms were brought into the laboratory of the Central Department of Botany, Tribhuvan University, Kathmandu Nepal for identification.

2.3.2 Identification

The mushroom species were identified based on their key morphological characteristics. The expertise from the Plant Pathology unit of The Central Department of Botany assisted in identifying samples. The published literatures (Tibuhwa et al., 2010; Adhikari and Durrieu, 2012; Guzmán et al., 2013) were also used in the identifying process. Online resources like catalogueoflife.org and indexfungorum.org were also used to identify the species names.

2.3.3 Preparation of extracts

All the laboratory work was done in Laboratory of Catalysis and Frontier Molecules, NAST, Khumaltar, Lalitpur. Mushrooms extract preparation was done by the Soxhlet extraction method following the protocol by Tesfaye & Tefera, 2017 with some modifications. Collected mushroom samples were first cleaned and left for shade drying for about 6 to 7 days. Then mushroom samples were ground into a coarsely fine powder sample with the help of a domestic grinder. The requisite-sized thimbles were made from filter paper. Twenty five gram of the mushroom powder sample was weighed and put inside the thimble and covered at the bottom as well as from the top. Then thimbles with the samples were put inside the Soxhlet. Then flat bottom flasks were kept on the Heating mantle and all the Soxhlet apparatus were joined to perform the task. Then about 300 mL of solvent was added from the top of the thimble. Then turn on the chiller for condensation and also the heating mantle to start the process. Then the process was continued for about 8-12 hours, or the extraction process was done until the colourless liquid started to fall from the thimble. Then all the aliquots were collected in the Flat Bottom flasks and subjected to evaporating with the help of a Rotary evaporator. Then semisolid extracts were poured into vials and all the vials with the extracts were kept inside the Desiccator to completely dry out the extracts. Then the vials with extracts were kept inside the fridge at 4°C until further analysis.

At first, extraction was done with Hexane. Then the marc of hexane extraction was subjected to extraction with methanol whose mark was again extracted with water following the above Soxhlet extraction process. The Figure 4 below shows the overall extraction process.

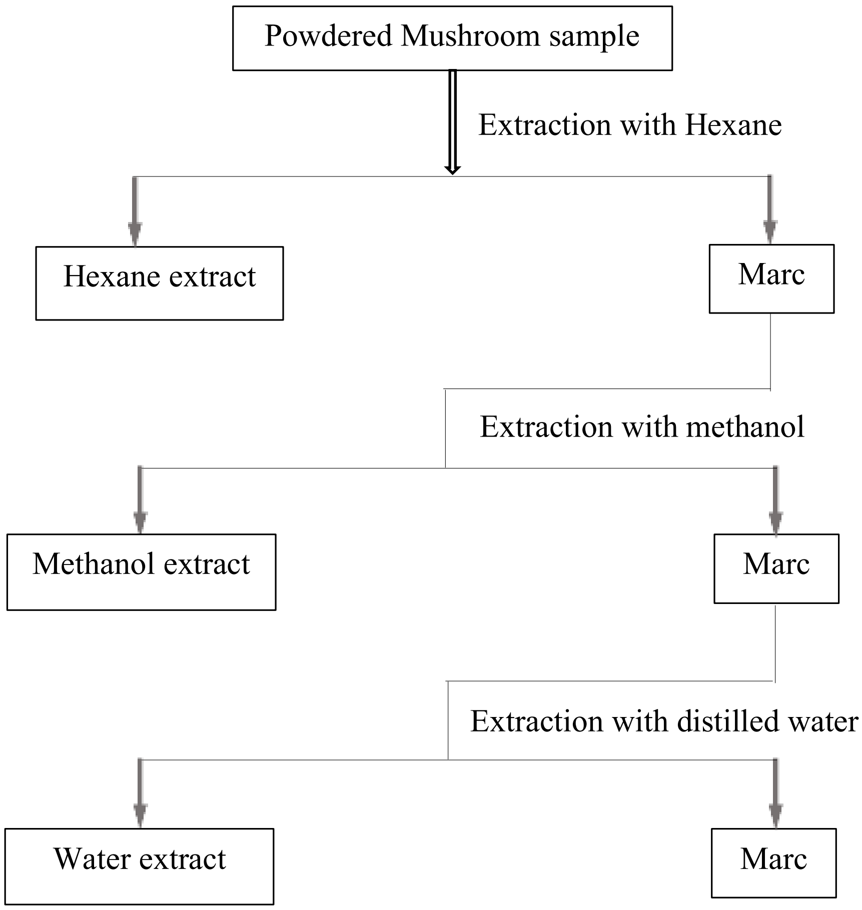


Figure 4: Flow chart showing extraction process

2.3.4 Estimation of extracts yield

The yield percentages of different extracts were calculated by using the given formula.

$$\text{Percentage of yield} = \frac{\text{Weight of extract (g)}}{\text{Weight of sample powder(g)}} \times 100$$

2.3.5 Phytochemical screening of mushroom samples of different extracts

A preliminary qualitative phytochemical screening was carried out on extracts of hexane, methanol, and water. The crude extracts obtained from the sequential extraction process were subjected to a variety of qualitative tests using previously described methods with some modifications for detecting the various phytoconstituents present (Ciulei, 1982; Harborne, 1998; Adebayo and Ishola, 2009, and Jagessar, 2017).

2.3.5.1 For hexane Extract

Alkaloids

By Dragendroff's Test

The extract was agitated vigorously with 2% HCl and then filtered. 3 drops of Dragendroff's reagent were added to the solution was treated with (3Drops). The formation of orange or red precipitate suggests the presence of alkaloids.

By Wagner's Test

In about 2 mL of the sample, a few drops (3 drops) of Wagner's reagent was added. The appearance of a reddish-brown precipitate indicates the presence of alkaloids.

Carotenoids

The extract was thoroughly shaken with chloroform ($CHCl_3$) and filtered. The solution was treated with conc. H_2SO_4. A blue colour at the interface indicates the presence of Carotenoids.

Steroids

The extract was dissolved in chloroform. A few drops of acetic anhydride and conc. H_2SO_4 were added to the solution. The presence of steroids is indicated by the production of dark pink, red, violet, blue, or green colors.

Terpenoids

The extract was dissolved in $CHCl_3$ and a few drops of conc. H_2SO_4 was added carefully to form a layer. A reddish-brown coloration at the interphase formed indicates a positive result for the presence of terpenoids. After shaking and allowing the stand, the appearance of a golden colour indicates the presence of triterpenes.

Coumarins

The extract was dissolved in 5 mL of hot water and filtered. The solution was divided into two test tubes. The first tube was taken as control and the content of the second tube was made alkaline by adding 10%NH_4OH drop by drop until pH 8. These tubes were observed under UV light. A blue fluorescence observed against the control tube suggests the presence of coumarins.

Anthraquinones

The extract was heated for a few minutes in 10% HCl, then filtered and chilled. An equal volume of Chloroform was to the filtrate. Then few drops of NH_3 (10%) were added and the solution was heated again. The appearance of a rose-pink color suggests the presence of anthraquinones.

2.3.5.2 For methanolic extract and eater Extract

Tannins and Polyphenols

At first, mushroom extracts were dissolved in water and heated in a water bath. The solution was filtered and 1% $FeCl_3$ were added (few drops) to the filtrate. A dark green, brownish green coloration indicates the presence of phenols.

Alkaloids

Same procedure as mentioned for hexane extract

Anthocyanosides

10% HCl was added to the extract and heated for few minutes. After cooling, Na_2CO_3 was added until it was basic to the litmus paper. The formation of a blue or brownish-green coloring suggests the presence of Anthocuanosides.

Anthracenosides

2 mL of ether, 1 mL of conc. H_2SO_4 and 1 mL of ammonia solution were added to the extract. The existence of anthracenosides is indicated by the formation of cherishes-red solution in the top layer.

Flavonoids

The extract was dissolved in about 2 mL of methanol and a piece of magnesium was added to the solution. A few drops of conc. HCl was added. The formation of pinkish-red coloration formation suggests the presence of flavonoids.

Cardiac Glycosides

The extract was dissolved in methanol (2ml) and treated with 1mL of methanolic KOH (1%) and 1mL of methanolic 3, 5-dinitrobenzoic acid (1%). Then, mixture was gently warmed. Appearance of purple color indicates the presence of Cardiac glycosides.

Saponins

The extract was thoroughly mixed with 5 mL of distilled water and brought to a boil. Then the solution was again shaken vigorously for a stable persistent froth. Appearance of a creamy mass of small bubbles of a 1cm layer suggests the presence of saponins.

Proteins

The extract was boiled with water and filtered. One drop of 2% copper sulphate solution was added to the filtrate. Again, 1mL of ethanol was added and few pieces potassium hydroxide pellets. The pink color formation indicates the existence of proteins in extract.

2.3.6 Total Phenol Content (TPC)

The Folin Ciocalteu method was used to determine TPC (Singleton et al., 1999) with a few changes. A stock solutions of both methanolic and water extracts were made in distilled water at a concentration of 2.5 mg/mL. A 75 g/L concentration of aqueous Na2CO3 solution was made. As a blank, distilled water was used. To create a calibration curve, standard Gallic acid solutions with concentrations of 100, 50, 25, 12.5, 6.2, 3.1, and 1.6 µg/mL were taken. In a 96-well microplate, 50 µL of prepared plant extracts solution was added and they were treated with 25µL of the Folin-

Ciocalteu reagent and 100 μL of aq. Na_2CO_3 solution. In the same way for the standard Gallic acid, 50μL of the Gallic acid of each concentration was put in the microwell plate and they were treated with the 25μL of Folin-Cioccalteu reagent and 100μL of aq. Na_2CO_3 solution. After adding all of the required solutions to the microplates, it was left for about one hour by covering with the lead. The absorbance was then determined using an Elisa microplate reader at 765 nm. The TPC value was calculated using a linear regression equation generated from the Gallic Acid calibration curve, and it was expressed as mg Gallic acid equivalents (GAE) per gram of dry extract. The formula for calculating the TPC value is given below.

C= cV/m., ………….. (1) (Singleton et al., 1999)

Where, C = total contents of compounds (mg GAE/g of dry extract)

c = the GA concentration (mg/mL)

V = the volume of extract (mL)

m = the weight of extract (g)

2.3.7 DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

The organic nitrogen radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) has a purple color and absorbs light between 515 and 520 nm. It has an unpaired valence electron at one atom of the nitrogen bridge (Vuolo et al., 2019). When an antioxidant is present, the intensity of the DPPH radical solution decreases and it turns yellow. This happens when the nitrogen atom in DPPH receives electrons from antioxidants.

With minor changes, the DPPH scavenging assay was conducted using the methodology described by Nemkul et al., 2022. The methanolic and water extracts were used in the assay. A 0.1nM concentration of DPPH radical solution was prepared by agitating 3.94 mg of DPPH in 100 mL of methanol at 0°C for a whole night in a Low Constant Temperature Bath. The DPPH solution was prepared under dark conditions. Stock solutions with a concentration of 2000 μg/mL of methanol extracts were prepared. A stock solution of the same concentration (i.e. 2000 μg/mL) of water extract was prepared in distilled water. In a 96-well microplate, the stock solution was diluted to achieve concentrations of 2000, 1500, 1000, 500, 100, 50, 25, and 10 μg/mL (total volume = 50 μL). The diluted solutions in a microplate were then treated with 250 μL of DPPH solution. 50 μL of Gallic acid solutions at concentrations of 50, 20, 10, and 5 μg/mL were used to create the linear curve for the positive control. Methanol was utilized as the blank. As a control, 250 μL of DPPH radical solution was mixed with 50 μL of methanol. After adding all of the required solutions in triplicates to the microplates, the microplates were kept in the dark for about 30 minutes. The absorbance was then measured using an Elisa microplate reader at 517 nm. The following formula was used to calculate the DPPH radical inhibition ability.

$$\text{DPPH inhibition } \% = 1 - \frac{\text{A(sample)} - \text{A (blank)}}{\text{A (control)} - \text{A(blank)}} \times 100 \qquad \text{(Nemkul et al., 2022)}$$

Where,

A (sample) = Absorbance of the Sample

A (blank) = Absorbance of the Blank

A (control) = Absorbance of the Control

The plotted graph of the DPPH Scavenging percentage against the extract concentrations was used to obtain the IC_{50} value. The amount of an antioxidant-containing material needed to scavenge 50% of the initial DPPH radicals is known as the IC_{50}.

2.3.8 ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay

The reaction in an ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) scavenging assay involves the reduction of the $ABTS^{\cdot +}$ by antioxidants present in the tested sample. The $ABTS^{\bullet +}$ is generated when of ABTS reacts with a strong oxidizing agent likea potassium persulfate. The resulting blue-green $ABTS^{\cdot +}$ has a characteristic absorbance at a certain wavelength (usually around 734nm) which can be measured spectrophotometrically (Vuolo et al., 2019).

A method described in Lalhminghlui and Jagetia, 2018 was followed to determine the ABTS scavenging activity with some changes. To make the potassium persulphate solution, 37.5 mg of potassium persulphate was mixed with 1 mL of distilled water. Next, 2.5 ml of distilled water was used to dissolve 9.5 mg of ABTS. To the 2.5ml of ABTS solution, 44 µl of Potassium persulphate solution was added to prepare the ABTS radical cation solution. The finally made solution was allowed to stand in the dark at room temperature for about 15 h with continuous stirring using a magnetic stirrer. The working solution for the assay was then prepared by mixing 1 mL of ABTS solution with 88 mL of 50% ethanol. The absorbance of the ABTS working solution was adjusted to 0.70 at 734nm by adding 50 % ethanol. Different concentrations of mushroom samples i.e. 2000, 1500, 1000, 500, 100. 50, 25, and 10 µg/mL were prepared for the assay. Gallic acid was taken as a standard. To obtain a linear curve of the positive control, Gallic acid solutions of the concentrations 100, 50, 25, 10, 5 µg/ml were used.

In 96-well plates, 50 µL of each concentration of the mushroom extracts were poured with the help of a micropipette and 250 µL of ABTS working solution was added to them. Similarly, for the Gallic acid standard 50 µL of different concentrations were poured into the same microplates, and 250µL of ABTS working solution was added to them. A solution of 50µL of methanol and 250 µL ABTS solution was used as control. 300 µL methanol was used as a blank. After adding all of the required solutions in triplicates into the 96-well microplates, the microplates left in the dark for about 10 min. Next, the absorbance was measured at 734nm using an Elisa microplate reader. The formula for ABTSi nhibition rate of the sample is given below.

$$\text{ABTS inhibition \%} = 1 - \frac{\text{A(sample)} - \text{A (blank)}}{\text{A (control)} - \text{A(blank)}} \times 100 \qquad \text{(Nemkul et al., 2022)}$$

Where,

A (sample) = Absorbance of the Sample

A (blank) = Absorbance of the Blank

A (control) = Absorbance of the Control

IC_{50} value was derived using the graph of the ABTS inhibition percentage vs extract concentration. The IC_{50} is the concentration of an antioxidant-containing substance necessary to inhibit 50% of the initial ABTS radicals.

2.4 Statistical Analysis

Three replicated samples were used for each bioassay. The data were reported as means ± standard error. The Shapiro-Wilk test was used to assess the normality of the datasets ($p>0.05$ considered data are approximately normally distributed). All data for methanol- and water-based extracts were compared independently using one-way

ANOVA to compare the mean TPC values between different mushroom samples. A pairwise comparison was completed using Duncan's multiple-range test. T-tests were employed for each mushroom species to compare the TPC values of methanol- and water-based extracts. The relationship between antioxidant activity and TPC was examined using the Pearson correlation coefficient. All analyses were carried out using RStudio and MS Excel.

3. RESULTS

3.1 Extracts Yield

The percentage of the yield of the mushroom sample varied with the solvent used in the extraction process. Among the three solvents used, the overall yield percentage was high with the methanol, and very little yield was found with the hexane. Water extract gave moderate amount of yield in comparison with hexane and methanol. Highest yield was found in *Termitomyces microcarpus* (4.12 % in hexane extract, 25.68 % in methanol extract, and 20.02 % in water extract) and very low yield was found in *Lentinula edodes* (0.64 % in hexane, 9.76 % in methanol extract and 5.24 % in water extract). The yield percentages of all six mushrooms were presented below in Table 1.

Table 1: Yield percentage of mushroom samples in different solvent extraction

S.N.	Mushroom Samples	Solvent		
		Hexane (%)	Methanol (%)	Water (%)
1	*Agaricus bisporus*	1.76	16.72	9.12
2	*Lentinula edodes*	0.64	9.76	5.24
3	*Pleurotus ostreatus*	1.72	16.84	13.24
4	*Scleroderma cepa*	0.8	10.2	6.04
5	*Termitomyces microcarpus*	4.12	25.68	20.02
6	*Termitomyces schimperi*	1.12	23.28	12.64

3.2 Qualitative phytochemical screening

3.2.1 Result of qualitative phytochemical screening of hexane extract

In the case of hexane extract, alkaloides, steroides, and terpenoides were present in all six mushroom samples. Carotenoids were present only in *L. edodes*, *P. ostreatus*, *T. schimperi,* and *S cepa*. Triterpenes were only present in *A. bisporus* and *T. schimperi*. The results of qualitative phytochemical screening of hexane extract of all mushroom samples are presented in Table 2.

Table 2: Phytochemical screening of hexane extract of different mushroom species

S.N.	Phytoconstituents	Mushroom Species	Hexane Extract
1	Alkaloids (Dragendorff test and Wagners test)	*Agaricus bisporus*	+
		Lentinula edodes	+
		Pleurotus ostreatus	+
		Scleroderma cepa	+
		Termitomyces microcarpus	+
		Termitomyces schimperi	+
2	Carotenoids	*Agaricus bisporus*	-
		Lentinula edodes	+
		Pleurotus ostreatus	+
		Scleroderma cepa	+
		Termitomyces microcarpus	-
		Termitomyces schimperi	+
3	Steroids (Liebermann-Burchard test)	*Agaricus bisporus*	+
		Lentinula edodes	+
		Pleurotus ostreatus	+
		Scleroderma cepa	+
		Termitomyces microcarpus	+
		Termitomyces schimperi	+
4	Coumarins (Fluorescence Test)	*Agaricus bisporus*	-
		Lentinula edodes	-

		Pleurotus ostreatus	-
		Scleroderma cepa	-
		Termitomyces microcarpus	-
		Termitomyces schimperi	-
5	Triterpenes	*Agaricus bisporus*	+
		Lentinula edodes	
		Pleurotus ostreatus	-
		Scleroderma cepa	-
		Termitomyces microcarpus	-
		Termitomyces schimperi	+
6	Terpenoids (Salkowski test)	*Agaricus bisporus*	+
		Lentinula edodes	+
		Pleurotus ostreatus	+
		Scleroderma cepa	+
		Termitomyces microcarpus	+
		Termitomyces schimperi	+
7	Anthraquinones (Borntiager test)	*Agaricus bisporus*	-
		Lentinula edodes	-
		Pleurotus ostreatus	-
		Scleroderma cepa	-
		Termitomyces microcarpus	-
		Termitomyces schimperi	-

Note: "+" indicates **Presence** and "-" indicates **Absence**

3.2.2 Phytochemical screening of methanol and water extract of different mushroom species

Alkaloids, polyphenols, cardiac glycosides, and saponins were present in both methanol and aqueous extract of six different mushroom samples. In the case of methanol extract, Flavonoids were moderately present in five mushroom samples but in *Lentinula edodes* samples it was almost absent. Water extract of all mushrooms didn't show any positive result for the Flavonoids test. The results of the preliminary phytochemical screening of six different mushroom samples in methanolic and water extract are given below in Table 3.

Table 3: Phytochemical screening of Methanol and Water extracts

S.N.	**Phytoconstituents**	**Mushroom species**	**Methanol Extract**	**Water Extract**
1	Alkaloids(Dragendorff test and Wagners test)	*A. bisporus*	+	+
		L. edodes	+	+
		P. ostreatus	+	+
		S. cepa	+	+
		T. microcarpus	+	+
		T. schimperi	+	+
2	Tannins and Polyphenol (Braymer test)	*A. bisporus*	+	+
		L. edodes	+	+
		P. ostreatus	+	+
		S. cepa	+	+
		T. microcarpus	+	+
		T. schimperi	+	+
3	Anthracenosides	*A. bisporus*	-	-
		L. edodes	-	-
		P. ostreatus	-	-
		S. cepa	-	-

		T. microcarpus	-	-
		T. schimperi	-	-
4	Anthocyanosides	*A. bisporus*	-	-
		L. edodes	-	-
		P. ostreatus	-	-
		S. cepa	-	-
		T. microcarpus	-	-
		T. schimperi	-	-
5	Flavonoids(Shinoda test)	*A. bisporus*	+	-
		L. edodes	-	-
		P. ostreatus	+	-
		S. cepa	+	-
		T. microcarpus	+	-
		T. schimperi	+	-
6	Cardiac glycosides (Kedd's Test)	*A. bisporus*	+	+
		L. edodes	+	+
		P. ostreatus	+	+
		S. cepa	+	+
		T. microcarpus	+	+
		T. schimperi	+	+
7	Saponins (Forth test)	*A. bisporus*	+	+
		L. edodes	+	+
		P. ostreatus	+	+
		S. cepa	+	+
		T. microcarpus	+	+
		T. schimperi	+	+

Note: "+" indicates **Presence** and "-" indicates **Absence**

The overall results of phytoconstituents present in mushroom samples are presented in figure 5.

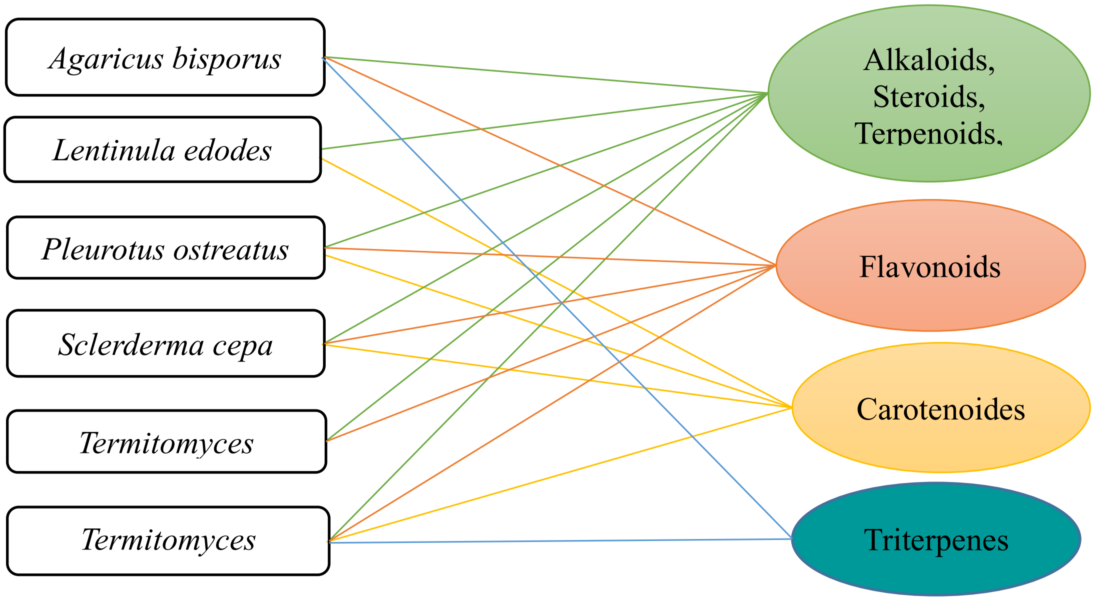


Figure 5: Phytoconstituents present in different mushroom samples.

3.3 Total Phenolic Content (TPC)

The Total Phenolic Content (TPC) was calculated for the methanolic and aqueous extract of different mushroom samples. The concentration of total phenolic in each extract was calculated using a linear regression equation obtained from the calibration curve of Gallic Acid (Figure 6).

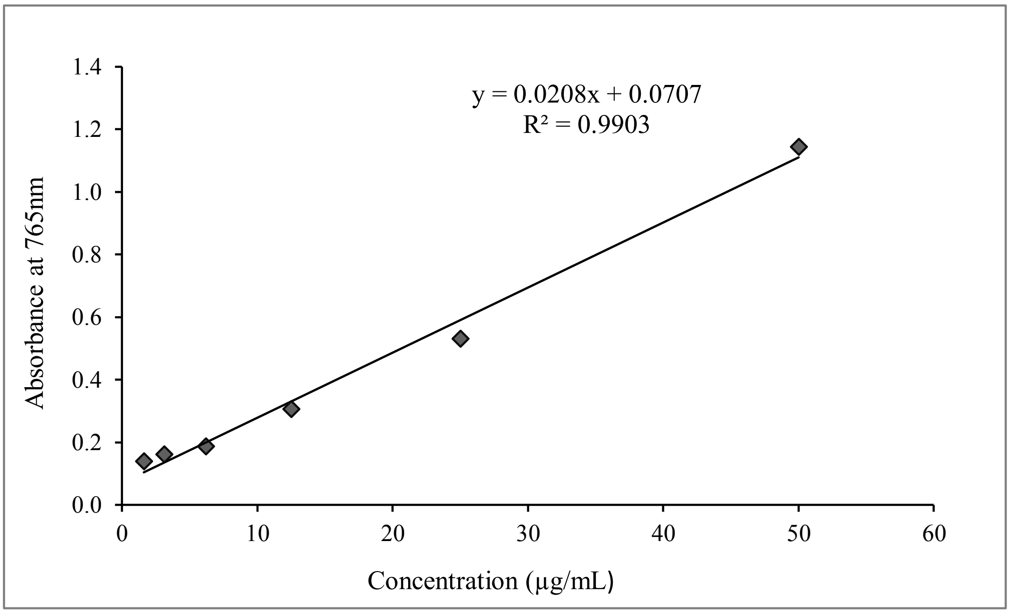


Figure 6: Gallic acid calibration curve

When comparing two different types of extraction, the water extract of cultivated and wild mushrooms showed a considerably higher phenolic content than the methanol extract except in wild species *Termitomyces schimperi*. In *T. schimperi*, TPC was higher in methanol extract than in water extract.

In the case of methanol extract, wild mushroom *Scleroderma cepa* has significantly higher phenolic content in comparison with other cultivated and wild mushroom species with the TPC value 29.94±4.61 mg GAE/ g dry extract. The second richest species regarding their total phenol content was that of *Termitomyces microcarpus* (25.37±3.38 mg GAE/g dry extract) followed by *T. schimperi* (23.44±1.23 mg GAE/ g dry extract). Regarding commercially cultivated mushrooms, the highest phenolic content was found in *Pleurotus ostreatus* (21.13±1.0338 mg GAE/g dry extract) followed by *Lentinula edodes* (14.21±0.68 mg GAE/g dry extract) and *Agaricus bisporus* (11.60±0.80 mg GAE/g dry extract) (Table 4).

In the case of water extract the highest TPC value was also found in *S. cepa* (36.57±0.81mg GAE/ g dry extract), and the second highest total phenol content was found in *T. microcarpus* (29.66±0.48 mg GAE/ g dry extract) followed by *A. bisporus* (28.93±0.13 mg GAE/ g dry extract). *T. schimperi* (19.19±0.17 mg GAE/ g dry extract) and *Lentinula edodes* (19.25±0.33 mg GAE/ g dry extract) have almost similar phenolic content values. The lowest phenolic content value was found in *P. ostreatus* (16.98±1.61mg GAE/ g dry extract) (Table 4).

Table 4: TPC of different mushroom samples in different solvent

Source	**Mushroom species**	**TPC in Methanol extract (mg GAE/g dry extract)**	**TPC in Water extract (mg GAE/g dry extract)**
Cultivated	*A. bisporus*	$11.60±0.80^{d}$	$28.93±0.13^{b}$
	L. edodes	$14.21±0.68^{cd}$	$19.25±0.33^{c}$
	P. ostreatus	$21.13±1.03^{bc}$	$16.98±1.61^{c}$
Wild	*S. cepa*	$29.94±4.61^{a}$	$36.57±0.81^{a}$
	T. microcarpus	$25.37±3.38^{ab}$	$29.66±0.48^{b}$
	T. schimperi	$23.44±1.23^{ab}$	$19.19±0.17^{c}$

Note: Values are given in means±standard error (n=3). For each sample, means with different letters denote significant differences at P<0.05.

The result shows that there is a significant difference between the mean TPC values of different samples at the level of $p<0.05$. The TPC values of different extracts are summarized below in Table 4.

An independent sample t-tests were employed for each mushroom species to compare the TPC values of methanol and water-based extracts. Statistically, it was found that there is a significant difference between the TPC values in methanol and water extracts of the samples *L. edodes*, *A. bisporus*, and *T. schimperi*. Other species did not show any significant difference in their total phenolic content values in methanol and water extracts. The results of t-test are shown in Table 5.

Table 5: T-test between the TPC values of methanol and water extracts

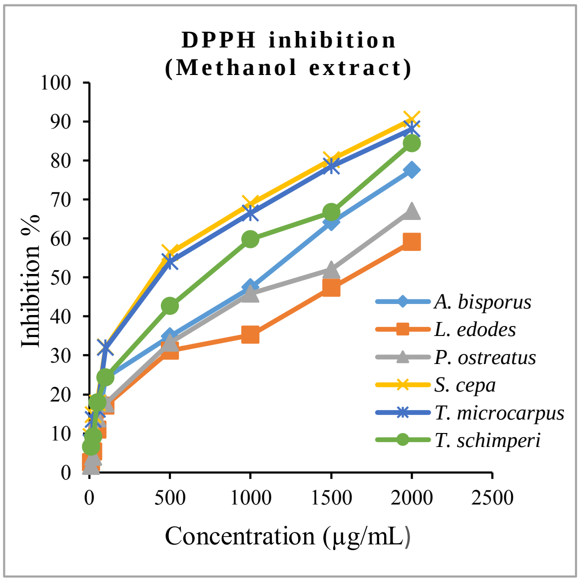
S.N.	Species	Methanol extract/water extract	p-value
1	*A. bisporus*	Independent t-test	$P<0.05$
1	*L. edodes*	Independent t-test	0.002653
3	*P. ostreatus*	Independent t-test	0.09564
4	*S. cepa*	Independent t-test	0.2296
5	*T. microcarpus*	Independent t-test	0.2778
6	*T. schimperi*	Independent t-test	0.02654

Note: difference is significant at the level of $p<0.05$

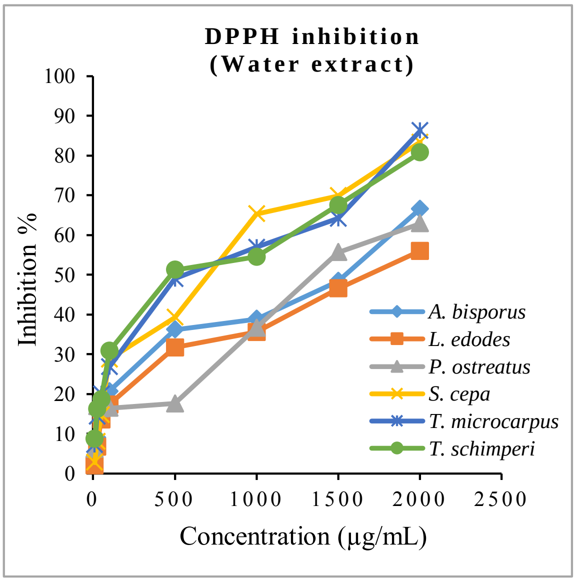
3.4 Antioxidant activity

3.4.1 DPPH scavenging activity

The inhibition percentage of DPPH was calculated by taking several concentrations per sample for both methanol and water extracts. The scavenging effects of both methanol and water extracts of mushroom samples on DPPH radical increased with the increasing concentrations. The concentrations of the samples ranged from 10 to 2000 µg/mL. At a sample concentration of 2000 µg/mL, wild edible mushrooms (*Scleroderma cepa*, *Termitomyces microcarpus*, and *Termitomyces schimperi*) have a higher inhibition percentage than commercially cultivated mushrooms (*Agaricus bisporus, Lentinula edodes*, and *Pleurotus ostreatus*). This was observed in both methanol and water extracts. Among all, the highest inhibition was observed in *S. cepa* (90.5±0.20 % in methanol extract and 83.39±0.73 % in water extract). The DPPH inhibition percentages



A



B

Figure 7: DPPH scavenging activity. (A) DPPH inhibition by methanolic extracts (B) DPPH inhibition by water extracts

of the samples are given in Appendix 1 and Appendix 2. And, percentages of all samples in both methanol and water extracts were compared using graphs, which are shown in Figure 7.

The scavenging ability of mushroom extracts against DPPH free radicals was also measured in terms of IC_{50}. A lower IC_{50} value implies that the extracts are more effective as DPPH radical scavengers. The IC_{50} values are shown in Table 6.

Table 6: IC_{50} values for various extracts in inhibiting the DPPH radical

Source	**Mushroom Species**	**DPPH Scavenging activity**	
		IC_{50} of Methanol extract (mg/mL)	**IC_{50} of Water extract (mg/mL)**
Cultivated	*A. bisporus*	1.122	1.401
	L. edodes	1.573	1.636
	P. ostreatus	1.366	1.45
Wild	*S. cepa*	0.744	0.935
	T. microcarpus	0.787	0.92
	T. schimperi	0.953	0.925

In the instance of methanol extract, the *Scleroderma cepa* sample had the lowest IC_{50} value of 0.774mg/mL, indicating that it has the potential to inhibit the DPPH radical by 50% at that concentration. All three wild species have lower IC_{50} values than other cultivated mushrooms. A similar case was discovered in the water extract, although in this case, all three wild mushrooms have nearly identical IC_{50} values. In water extract, the mushroom *Termitomyces microcarpus* had the lowest IC_{50} value of 0.920 mg/mL.

Except for *Termitomyces schimperi*, all other mushrooms had a lower IC_{50} value in methanol extract than in water extract. Although there was no major difference in results between the methanol and water extracts of *T. schimperi*. The IC_{50} values for all species are compared and displayed in the bar graph below (Figure 8).

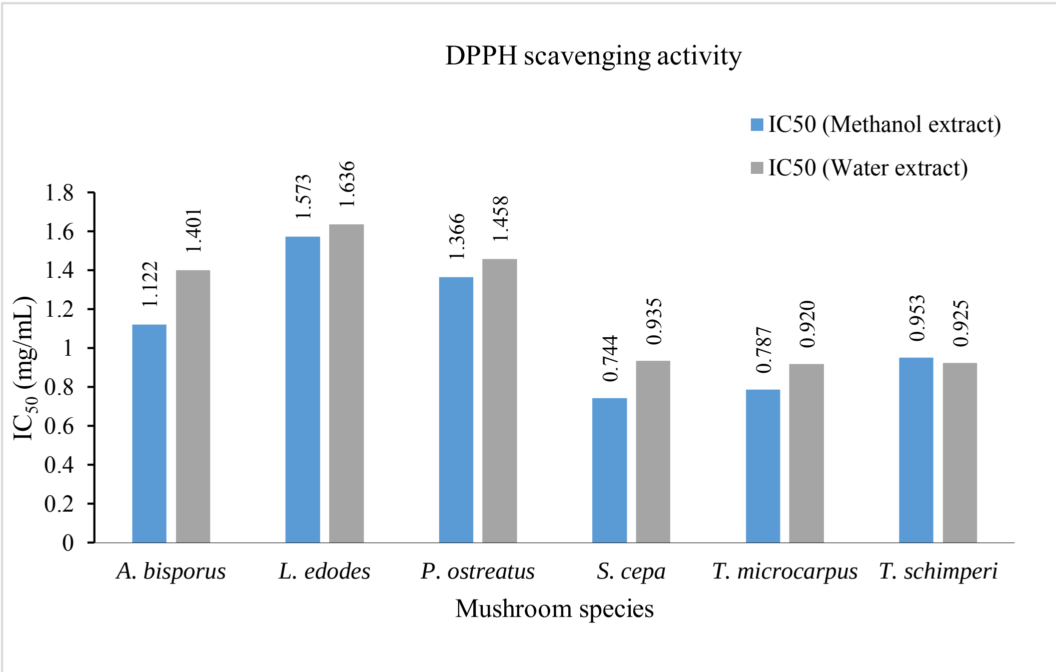


Figure 8: IC_{50} values of mushroom species in inhibiting DPPH in methanol and water extracts

Standard Gallic acid was also tested for its DPPH scavenging activity. IC_{50} of Gallic acid solution was found to be 0.015mg/mL. A linear curves obtained from values of DPPH radical inhibition percentage and concentrations of Gallic acid is given in Figure 9.

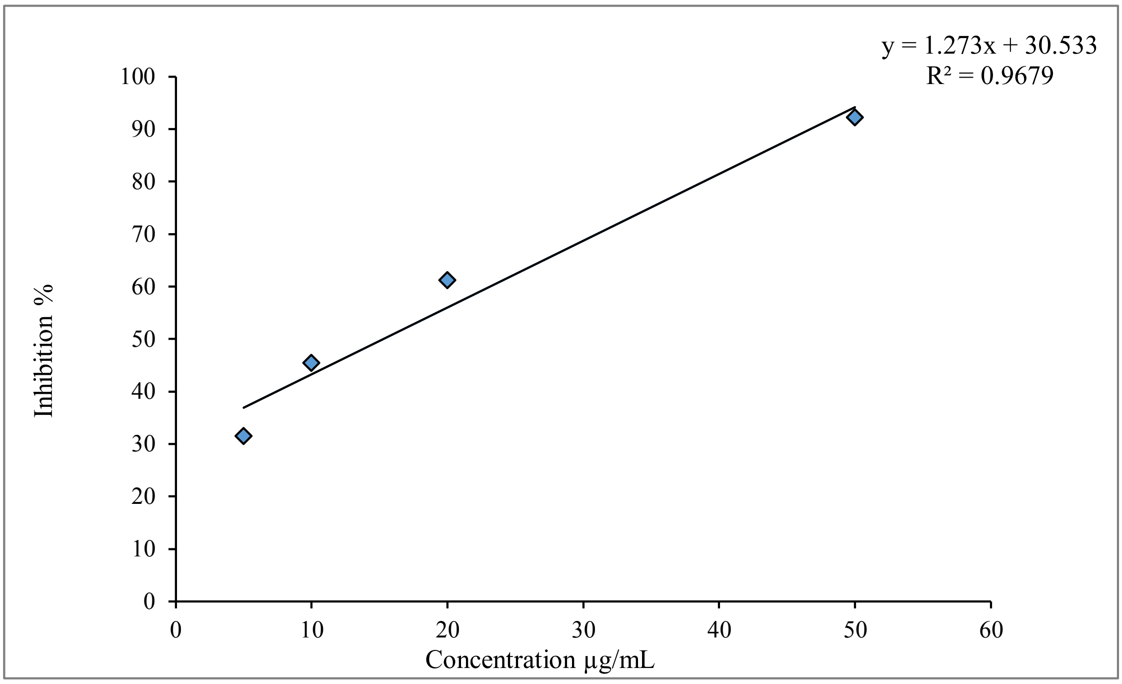
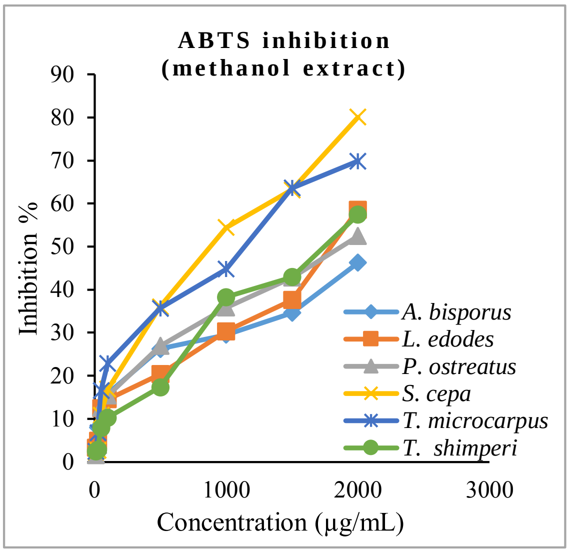


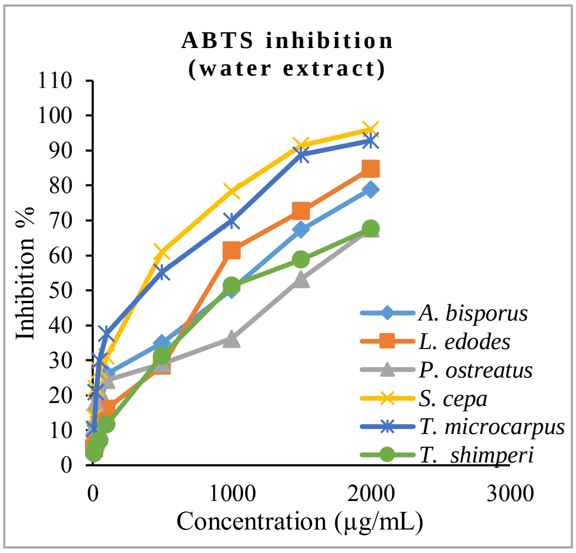
Figure 9: DPPH inhibition capacity of Gallic Acid

3.4.2 ABTS scavenging activity

ABTS scavenging assay was also carried out for both water and methanol extract of different samples. The scavenging effects of mushroom extracts in methanol and water on ABTS radicals increased with concentration. The concentration ranged from 10-2000 µg/mL. *Scleroderma cepa* inhibited the most at the maximum concentration of 2000µg/mL (80.06±2.53% in methanol extract and 96.09±1.42% in water extract). *Termitomyces microcarpus* is the second-highest ABTS inhibitor in both water and methanol extracts. In terms of ABTS scavenging activity, water extracts exhibit more inhibition than methanol extracts. Compared to *Termitomyces schimperi* and other cultivated mushrooms, *Lentinula edodes* had a higher inhibition percentage (58.56±1.26% in methanol extract and 84.7±4.89% in water extract). The details of ABTS inhibition activity of all mushroom species in both methanol and water extract are given in Appendix 3 and Appendix 4. An inhibition percentages of all mushrooms in methanol and water extract were compared through graphs and shown in Figure 10.



A



B

Figure 10: ABTS radical scavenging activity. (A) ABTS inhibition by methanolic extracts (B) ABTS inhibition by water extract.

The potential of mushroom mushroom extracts to scavenge ABTS radicals was also assessed through the IC_{50} method. IC_{50} value is the concentration of extract that inhibits the ABTS radical by 50 %. A lower IC_{50} indicates that the extracts are more effective as ABTS radical scavengers. The IC_{50} values of different samples in different extracts are presented in Table 7.

Table 7: IC_{50} values for various extracts in inhibiting the ABTS free radical

Source	**Mushroom Species**	**ABTS Scavenging activity**	
		IC_{50} of Methanol extract (mg/mL)	**IC_{50} of Water extract (mg/mL)**
Cultivated	*A. bisporus*	2.175	1.053
	L. edodes	1.796	0.991
	P. ostreatus	1.766	1.364
Wild	*S. cepa*	1.09	0.583
	T. microcarpus	1.195	0.63
	T. schimperi	1.627	1.226

In terms of ABTS radical scavenging activity, all six mushroom samples had lower IC_{50} values in water extracts than in methanol extracts. It implies that the mushroom samples exhibited better inhibition when extracted with water. *Scleroderma cepa* was the most potent inhibitor of ABTS radical, with a minimal IC_{50} value of 1.09 mg/mL in methanol extract and 0.583 mg/mL in water extract. Another wild edible mushroom, *Termitomyces microcarpus*, also demonstrated higher inhibition with an IC_{50} value of 1.195 mg/mL in methanol extract and 0.63 mg/mL in water extract. Among the other wild and cultured mushrooms included in the study, cultivated *Lentinula edodes* (1.796 mg/mL in methanol extract and 0.991 mg/mL in water extract) performed the best. *Pleurotus ostreatus* was the weakest inhibitor with the highest IC_{50} value. The bar diagram below shows how the values of the methanol and water extracts differ (Figure 11).

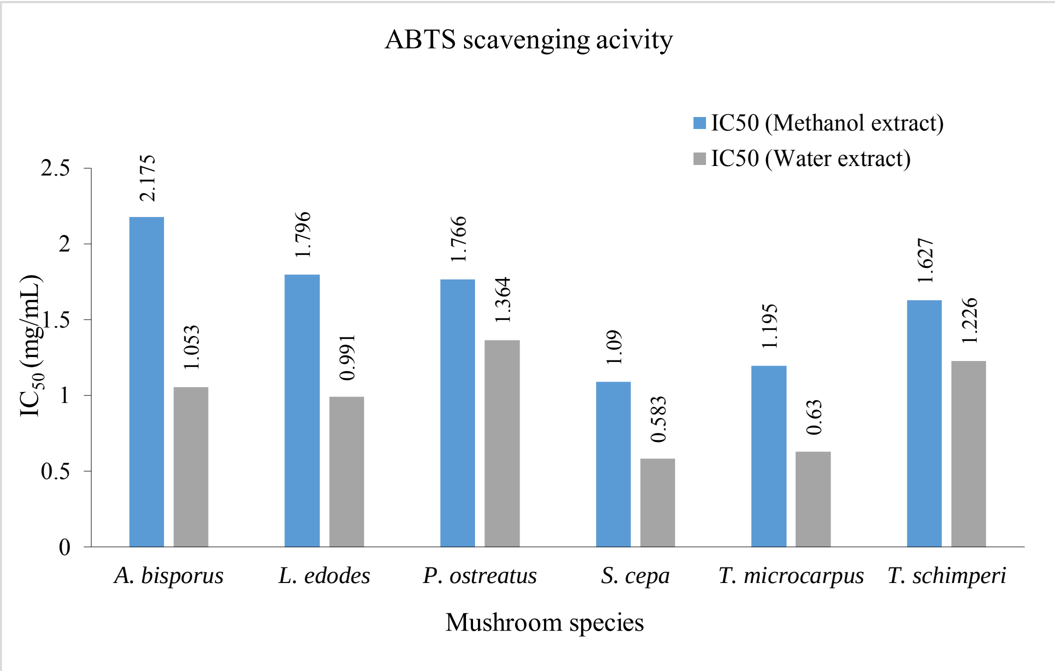


Figure 11: IC_{50} values of mushroom species in inhibiting ABTS in methanol and water extracts

Gallic acid was taken as a standard ABTS inhibitor. IC_{50} value Gallic acid in inhibiting ABTS radical was determined to be 0.018 mg/mL. IC_{50} value was calculated from the linear curve obtained from values of ABTS radical inhibition percentage and concentrations of Gallic acid which is shown in Figure 12.

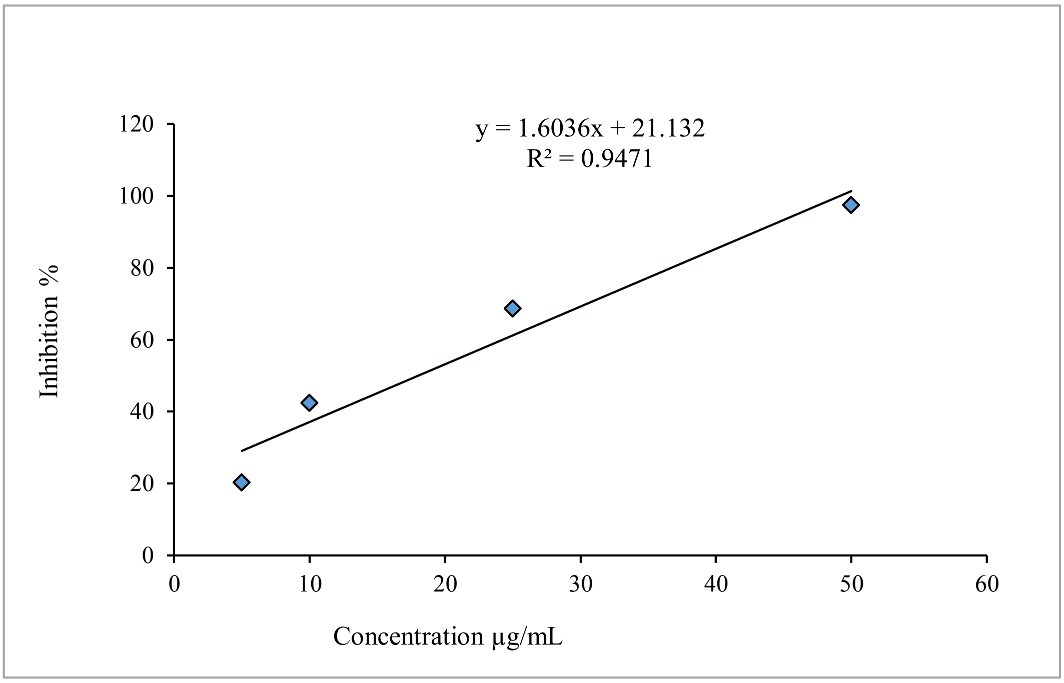


Figure 12: ABTS scavenging capacity of Gallic acid

3.4.3 Correlation between TPC and antioxidant activity

The Pearson correlation coefficient was used to determine the relationship between antioxidant activity and total phenolic content.

3.4.3.1 Correlation between TPC and DPPH scavenging activity

The correlation between total phenolic content and DPPH scavenging activity was investigated for both methanol and water extracts of all samples. TPC and IC_{50} values from the samples were used as variables. In terms of DPPH scavenging activity, both methanol and water extract did not exhibit a perfect association (Table 8).

The correlation coefficient (r) for methanol extract was -0.729, indicating a negative correlation between total phenolic content and DPPH scavenging capacity. This indicates that as TPC levels rise, the extract's IC_{50} value decreases, and *vice versa*. The lower the IC_{50} value, the more effective the sample. However, the p-value in this instance was 0.1002, which is above the standard significance level of 0.05, assuming no actual negative correlation between TPC content and IC_{50} value. Similar results were observed in water extract, with a correlation coefficient (r) of -0.5237 and a p-value of 0.2862, indicating that there is no actual negative correlation between total phenolic content and IC_{50} values.

3.4.3.2 Correlation between TPC and ABTS scavenging activity

The samples' IC_{50} values for blocking the ABTS radical and total phenolic content were used as variables. The correlation coefficient (r) for methanol extract is -0.9254, indicating a very significant negative linear association between total phenolic content and the samples' IC_{50}. This indicates that while TPC grows, IC_{50} decreases, and *vice versa*. The p-value (0.0081) is also less than 0.05. This indicates that the correlation between TPC and ABTS• scavenging capacity is statistically significant (Table 8).

In the case of water extract, the correlation coefficient value of -0.8606, with a p-value of 0.0278, indicates that there is a strong negative correlation between total phenolic content and sample IC_{50} value, which is also statistically significant because the p-value is less than 0.05.

These findings indicates that Phenolic present in the samples may have an important role in suppressing the ABTS radical. However, in terms of DPPH scavenging action, phenolic compounds may not have a substantial role in suppressing the DPPH radical. The Pearson correlation results are reported in the table 8 below.

Table 8: Pearson correlation between the TPC and IC50 values

Total Phenolic Content	**Pearson Correlation**		
		DPPH	**ABTS**
Methanol	r value	-0.729	-0.925
	p value	0.1	0.0081
Water	r value	-0.523	-0.86
	p value	0.286	0.027

4. DISCUSSION

4.1 Phytochemical extraction

The selection of an extraction technique and a suitable solvent is a crucial step in the phytochemical extraction process since biologically active chemicals are found in extremely minute amounts in plants (Ngo et al., 2017). The temperature, duration, raw material particle size, phytochemical makeup, solvent characteristics, and extraction technique may have a significant impact on the extraction's efficiency (Truong et al., 2019; Mosic et al., 2020)

In the present study, hexane, methanol, and distilled water were used in the extraction process. Results showed that extraction yield was different for different solvents. Among the three solvents used, the yield was higher in methanol extracts in all species. This result indicates that methanol can be considered the best solvent in terms of yield in phytochemical extraction. This result was also supported by various studies (Anokwuru et al., 2011; Adam et al., 2019; Truong et al., 2019). Water extracts also gave a higher yield than hexane extracts. After methanol, water can also be a very good solvent for extraction in terms of yield value. A very minimum yield was found in hexane extracts. However, according to the nature of solvents, different solvents extract different phytoconstituents.

Hexane is a non-polar solvent that is often used for extracting non-polar compounds such as fatty acids, carotenoids, and essential oils. Methanol is a polar solvent, however, methanol also has a non-polar methyl (CH_3) group attached to the polar hydroxyl group. Because of methanol's dual nature having both polar and non-polar characteristics, methanol may effectively dissolve a wide range of substances including both polar and non-polar substances. Water is a polar solvent with a high polarity index, which can extract various polar substances from the sample. As a result of different properties of the solvents, there was a variation in extraction yield across the three different solvent extraction in this study.

The extraction methods applied may also affect on the yield value of the mushroom samples. Soxhlet extraction is an automatic and continuous extraction process with higher extraction efficiency and lower solvent and time requirements than other methods such as maceration and percolation. Therefore, high yield percentage in the various samples was also attributed to the extraction process used in this research. However, the high temperature and long extraction time in Soxhlet extraction increases the possibility of thermal destruction of bioactive compounds (Li et al., 2008). As a result, more care should be taken throughout the Soxhlet extraction procedure.

4.2 Preliminary phytochemical screening

Since the different solvents extract different phytochemicals present in the sample, preliminary tests were done separately for non-polar solvent- hexane and polar solvents methanol and water. In this study, hexane extract includes Alkaloids, Steroids, Terpenoids, Carotenoids, and Triterpenes. Alkaloids, Steroids, and Terpenoids were present in all six mushroom samples. Carotenoids were present only in *L. edodes, P. ostreatus, T. schimperi, and S. cepa* and absent in *Agaricus bisporus* and *Termitmyces microcarpus*. Triterpenes were only present in *A. bisporus* and *T. schimperi*.

Because the water and methanol are polar in nature, similar experiments were performed for the preliminary phytochemical test. The preliminary test revealed that the methanol and water extracts included phytoconstituents such as Alkaloids, Polyphenols, Cardiac glycosides, Flavonoids, and Saponins. Alkaloids, Polyphenols, Cardiac glycosides, and Saponins were found in both methanol and water extract of six different mushroom samples. From the above experiment results, it was observed that there are no vast differences in phytoconstituent content in cultivated and wild mushrooms.

Many researches also have reported that cultivated mushroom species such as *A. bisopus, L. edodes,* and *P. ostreatus* include several bioactive chemicals such as flavonoids, phenolic compounds, tannin, saponin, alkaloids, and steroids (Jain and Choudhary, 2012; Edet et al.,2016; Rahimah et al., 2019). Various studies support that *Termitomyces* mushrooms are rich in bioactive compounds like alkaloids, steroids, terpenoids, saponins, and glycosides (Loganathan et al., 2010; Aryal and Budhathoki, 2013; Tharu et al., 2022). According to a study conducted by Shrestha et al., 2023, *Scleroderma cepa* is rich in protein and carbohydrates while low in fat content. This study supports that *S. cepa* is also rich in many phytoconstituents. Thus, it can be said that the wild edible and grown mushrooms used in the study are rich in phytoconstituents, which might be highly effective in the treatment of a variety of disorders.

4.3 Total Phenolic Content (TPC)

Because of their phenolic nature, polyphenols are generally hydrophilic and are therefore extracted using polar organic solvents like methanol, ethanol, and water. (Tsao, 2010). In the present study, methanol and water extracts of different mushroom samples were analyzed for Total Phenolic Content (TPC).

This study found that *S. cepa* is the richest species in terms of high Phenolic content value (29.94±4.61 mg GAE/ g dry extract in methanol extract and 36.57±0.81mg GAE/ g dry extract in water extract).

T. schimperi was the weakest wild edible species in terms of TPC content. There are various studies on the TPC of *Termitomyces* species. *T. albuminosus*, *T. eurhizus,* and *T. robustus* were found to be rich in TPC (Tharu et al., 2022). Total phenols were also detected in considerable amounts in *T. heimii* (Johnsy and Kaviyarasan, 2014) and *T. clyeatus* (Mitra et al., 2016). It indicates that *Termitomyces* species are important wild edible mushrooms with high phenolic content.

In the present study, the TPC value of *A. bisporus* in methanol extract was 11.60±0.80 mg GAE/g dry extract, and water extract was 28.93±0.13 mg GAE/g dry extract. However, according to previous studies conducted by researchers, the TPC value of cultivated *A. bisporus* in water extract was 6.43 mg GAE/g dry extract (Zalewska et al., 2017), and in ethanol extract was 9.48±.25 mg GAE/g dry extract (Jegachandran et al., 2024). This shows that there is a variation in TPC values according to the types of solvent used in the extraction process. The cultivation process may also affect the variation in the TPC value in cultivated *A. bisporus.*

Jagathchandran et al., 2024 reported that the water extract of *L. edodes* exhibited a TPC value of 4.94 mg GAE /g dry extract. However, in this study, the TPC value of the water extract of *L. edodes* was higher (19.25±0.33 mg GAE/g dry extract). This variation in TPC content may be due to the cultivation methods applied.

Yilmaz et al., 2017 reported that the cultivated mushroom *P.ostreatus* has a TPC value of 1.672±0.003 mg GAE/g dry extract on walnut sawdust substrate and 1.073±0.028 mg GAE/g dry extract on peanut wastes. This indicates that substrate composition affects the TPC content in mushroom species.

The result of the study showed that methanolic extract of selected wild mushrooms have greater TPC values than cultivated mushrooms. Genetic variations, environmental conditions, and cultivation practices could be the causes of this. In their native environments, wild mushrooms thrive in a variety of conditions such as soil composition, climate, exposure to sunlight, and the presence of other organisms. These ambient factors may influence the synthesis of phenolic compounds in wild mushrooms.

In carefully regulated settings with optimal growth conditions for temperature, humidity, and light, mushrooms are cultivated. In comparison to wild mushrooms, these regulated circumstances might produce phenolic content variations. The phenolic content of mushrooms can be influenced by the cultivation method itself. For example, the substrate used for cultivation, such as straw, sawdust, or compost can influence the availability of nutrients and phenolic precursors for the mushrooms. In addition, the production of phenolic compounds in mushrooms may be impacted by the application of pesticides, fertilizers, and other agricultural inputs during cultivation. Finally, the nutrients and secondary metabolites in the sample may potentially be impacted by the harvesting and storage processes.

The outcome of this study demonstrates that there is a significant difference between the TPC values within the various samples. Numerous factors, such as genetic variability, environmental influences, substrate specialization, species-specific metabolism, and interactions with microbes, could be responsible for the variations in TPC values within the species.

4.4 Antioxidant activity

4.4.1 DPPH radical scavenging assay

In this study, in both methanol and water extracts, all three wild species (*S. cepa*, *T. microcarpus*, and *T. schimperi*) have lower IC_{50} values than the other three commercially grown mushrooms (*A. bisporus L. edodes,* and *P. ostreatus*). In methanol extract, *Scleroderma cepa* had the lowest IC_{50} value (0.744 mg/mL), and in water extract, *Termitomyces microcarpus* had the lowest IC_{50} value of 0.920 mg/mL. This result implies that wild edible mushrooms have a higher potential to scavenge the antioxidants. A higher antioxidant capacity may linked to the diverse group of potent antioxidants present in the mushroom samples. Wild mushrooms grow in diverse natural habitats which expose them to several environmental stresses like temperatures, sunlight, predator organisms, etc. This can make the wild mushrooms potent antioxidants.

There is little information available about the genus *Scleroderma* in terms of phytochemical properties and biological activities, particularly antioxidant activity. This study found that the *S. cepa* was the strongest inhibitor of DPPH. Filho et al., 2022 reported that the IC_{50} value of *Scleroderma verrucosum* was 0.00597±0.91 mg/mL. This value is much lower than the IC_{50} value of *S. cepa* (0.744 mg/mL in methanol extract and 0.935 in water extract) in the present study. This indicates that the various *Scleroderma* species can be potent antioxidants-containing species.

There were various studies conducted on the phytochemical characteristics and antioxidant potential of the *Termitomyces* species. Obodai, 2014 reported that IC_{50} value of 4.78±0.05 mg/mL in *T. robustus*. Johnsy and Kaviyarasan, 2014 reported that IC_{50} value of 12.26±0.72 mg/mL in *T. heimii*. Tharu et al., 2022 reported that the IC_{50} values of *T. albuminosus*, *T. eurhizus*, and *T. robustus* were 0.7206 mg/mL, 0.71765 mg/mL, and 0.7166 mg/mL respectively. This study reported that the IC_{50} values of *T. microcarpus* and *T. schimperi* were 0.953 mg/mL and 0.953 mg/mL respectively. Mitra, 2014 reported that the IC_{50} value *T. microcarpus* was 1.98±02 mg/mL. This IC_{50} value is higher than the value reported in this study. This deviation in results might be due to the environmental conditions, habitat, and experiment procedure applied during research work.

One of the studies conducted by Kruzelyi et al., 2020 showed that the *A. bisporus*, *P. ostreatus*, and *L. edodes* had IC_{50} values of 0.151±0.001mg/mL, 0.246±0.004, and 0.141±0.001 respectively. However, in this study, IC_{50} values of these mushrooms were higher than 1mg/mL in both methanol and water extracts. This variation in the results may be due to the growing conditions such as substrates used, temperature, light, fertilizers used, and storage conditions of the mushrooms. Extraction methods may also be the reason for the variation in the results.

In the present study, IC_{50} of standard Gallic acid (GA) was much lower (0.015 mg/mL) than the IC_{50} of the samples, it indicates that Gallic acid is more potent in inhibiting DPPH than the mushroom samples. It may be due to Gallic acid is more pure than the samples. Impurities in the samples could reduce their effectiveness, resulting in higher IC_{50} values.

4.4.2 ABTS radical scavenging assay

In this study, the water extract of *Scleroderma cepa* was the strongest ABTS radical inhibitor with the lowest IC_{50} value of 0.583mg/mL. This result was close to the value reported by Vamanu and Nita, 2012 on a wild edible mushroom, *Boletus edulis* which had the IC_{50} value of 0.47±0.02 mg/mL. Two wild edible mushrooms, *Leccinum pseudoscaber,* and *Lactarious volumus* exhibited the IC_{50} value of 5.034±0.05 mg/mL and 16.394±0.475 mg/mL respectively (Dimitrijevic et al., 2015). This shows that wild edible mushrooms can be potent antioxidant foods.

The water extract of *T. microcarpus* was the strong ABTS radical inhibitor with the IC_{50} value of 0.63 mg/mL. However, this value is much higher than the value reported by Mondal et al., 2016 on water extract of *T. clypeatus* (0.0643mg/mL). The drying procedure of mushroom samples, extraction process may be the region of differences in values in same samples

Among cultivated mushrooms, water extract of *Lentinula edodes* was the strong inhibitor of ABTS radical with a value of 0.991 mg/mL. According to Mircea et al., 2015, *Agaricus bisporus* and *Pleurotus ostreatus* had an IC_{50} value of 0.226.60±2.97 mg/mL and 0.276.19±2.61 mg/mL respectively. But in this study, IC_{50} values of *A. bisporus and P ostreatus* was more than 1 mg/mL in both methanol and water extract. This variation in result may be due to the substrate composition of cultivation, drying of mushroom samples, solvents used in the extraction process, and the extraction methods applied during the extraction.

In ABTS radical scavenging assay, IC_{50} values of all species were lower in water extract than in methanol extract. It indicates that water extraction is more effective than methanol extraction in the case of ABTS radicals inhibition. Unlike in DPPH scavenging assay, all the wild edibel mushrooms didn't show lower IC_{50} value than the cultivated mushrooms. Only wild *S. cepa* and *T. microcarpus* exhibited lower IC_{50} values than the cultivated mushrooms. The differences may be the presence of diverse antioxidants in the samples, which interact differently with different radicals. (Shah and Modi, 2015). As in DPPH assat, standard antioxidant, Gallic acid also had a significantly lower IC_{50} value than the samples for ABTS scavenging activity.

4.5 Correlation between Total Phenolic Content and Antioxidant Activity

In the DPPH assay, there was no significant connection between TPC and IC_{50} values. But, in the case of ABTS inhibition activity, there was a significant negative correlation between the Total Phenolic Content and IC_{50} values of both methanol and water extracts of the samples. This indicates that while TPC grows, IC_{50} decreases, and *vice versa*. Vamanu and Nita, 2012 also mentioned that the Total phenols exhibited the strongest correlation with IC_{50} values of antioxidant properties. Loganathan et al., 2010 reported that there was a notably negative correlation established between the phenols and EC_{50} values of antioxidant activities.

In the antioxidant activity of phenolics, hydrogens and electrons are donated by hydroxyl groups resulting in the formation of stable radicals. The types of phenolics present and stability of radicals used in the assay may vary the inhibition capacity of the samples. Thus, the position and the hydroxylation number correlate with the antioxidant capacity of the phenolic present in the sample. Different phenolic compounds may have varying degrees of antioxidant capacity.

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study concluded that mushroom samples produced a higher yield with methanol than with the solvents hexane and water. Both wild edible and cultivated mushrooms taken in this study tend to contain phytochemicals like Alkaloids, Steroids, Terpenoids, Polyphenols, Cardiac glycosides and Saponins. Methanolic extract of all three wild edible mushrooms had higher TPC than the cultivated mushrooms.

The antioxidant assay revealed that majority of wild mushrooms are strong inhibitors of free radicals like DPPH and ABTS with the lowest IC_{50} values than the cultivated mushrooms. Wild mushrooms grow in diverse natural habitats which expose them to several environmental stresses like temperatures, sunlight, predator organisms, etc. This make the wild mushrooms potent antioxidants.

The significant negative correlation was observed between the Total Phenolic content and ABTS scavenging potential of the samples. But no significant correlation could observed in the DPPH radical scavenging assay. Differences in correlation results may be due to the difference in assay mechanisms, radicals' stability, specificity of antioxidants, and variability in sample composition.

Antioxidant compunds exist in both wild and cultivated mushrooms. Natural antioxidants can be used to substitute synthetic antioxidants, which will benefit public health. While some mushrooms may indeed be more potent than cultivated ones in terms of potent antioxidant compounds, it's essential to approach wild mushrooms with caution due to risks of toxicity and misidentification.

5.2 Recommendations

The recommendations based on this study are as follows.

- Further study should focus on identifying specific phytoconstituents present in these mushrooms sample.
- It is essential to screen the efficacy of the specific secondary metabolites as antioxidants *in vivo* using cell systems and animal models.
- Some mushrooms are found to be consumed only by specific localities whereas some other localities consider them as inedible. Thus, while consuming wild mushrooms, special care should be taken. The initial focus of the investigation should be on the toxicity of wild mushrooms.

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APPENDICES

Appendix 1: DPPH scavenging assay of methanol extract of mushroom samples

Species	Solvent	Conc. (µg/mL)	Abs.	Inhi. %	Mean±SE (%)	IC50 (mg/mL)
Agaricus bisporus	Methanol	10	0.604	2.76	2.47±0.86	1.122
			0.598	3.79		
			0.615	0.86		
		25	0.613	1.21	3.91±2.88	
			0.564	9.66		
			0.615	0.86		
		50	0.591	5.00	11.67±4.16	
			0.508	19.31		
			0.558	10.69		
		100	0.413	35.69	24.20±5.91	
			0.499	20.86		
			0.527	16.03		
		500	0.436	31.72	34.89±2.21	
			0.424	33.79		
			0.393	39.14		
		1000	0.344	47.59	47.41±4.03	
			0.386	40.34		
			0.305	54.31		
		1500	0.242	65.17	64.08±4.76	
			0.299	55.34		
			0.204	71.72		
		2000	0.178	76.21	77.47±0.87	
			0.161	79.14		

			0.173	77.07		
Lentinula edodes	Methanol	10	0.608	2.07	2.53±0.30	1.573
			0.602	3.10		
			0.606	2.41		
		25	0.593	4.66	5.34±0.53	
			0.583	6.38		
			0.591	5.00		
		50	0.556	11.03	10.86±3.04	
			0.527	16.03		
			0.588	5.52		
		100	0.516	17.93	17.13±0.57	
			0.527	16.03		
			0.519	17.41		
		500	0.42	34.48	31.21±1.73	
			0.454	28.62		
			0.443	30.52		
		1000	0.397	38.45	35.23±2.13	
			0.439	31.21		
			0.411	36.03		
		1500	0.324	51.03	47.30±3.65	
			0.325	50.86		
			0.388	40.00		
		2000	0.209	70.86	59.02±6.54	
			0.284	57.93		
			0.34	48.28		
Pleurotus ostreatus	Methanol	10	0.619	0.17	1.67±0.75	1.336
			0.605	2.59		

			0.607	2.24		
		25	0.619	0.17	3.91±3.40	
			0.558	10.69		
			0.615	0.86		
		50	0.551	11.90	13.79±2.99	
			0.506	19.66		
			0.563	9.83		
		100	0.598	3.79	17.64±6.93	
			0.479	24.31		
			0.476	24.83		
		500	0.423	33.97	33.22±0.83	
			0.422	34.14		
			0.437	31.55		
		1000	0.342	47.93	45.86±1.31	
			0.368	43.45		
			0.352	46.21		
		1500	0.292	56.55	52.01±3.05	
			0.352	46.21		
			0.311	53.28		
		2000	0.256	62.76	67.01±2.35	
			0.229	67.41		
			0.209	70.86		
Scleroderma cepa	Methanol	10	0.548	12.41	9.08±1.74	0.744
			0.572	8.28		
			0.582	6.55		
		25	0.531	15.34	14.71±1.28	
			0.549	12.24		

			0.524	16.55		
		50	0.51	18.97	17.70±0.80	
			0.516	17.93		
			0.526	16.21		
		100	0.449	29.48	32.07±1.60	
			0.417	35.00		
			0.436	31.72		
		500	0.298	55.52	56.32±0.89	
			0.283	58.10		
			0.299	55.34		
		1000	0.205	71.55	68.91±1.33	
			0.229	67.41		
			0.227	67.76		
		1500	0.142	82.41	80.17±1.15	
			0.164	78.62		
			0.159	79.48		
		2000	0.093	90.86	90.52±0.20	
			0.095	90.52		
			0.097	90.17		
Termitomyces microcarpus	Methanol	10	0.523	16.72	7.64±4.78	0.787
			0.587	5.69		
			0.617	0.52		
		25	0.521	17.07	13.51±1.78	
			0.552	11.72		
			0.552	11.72		
		50	0.511	18.79	16.09±1.77	
			0.523	16.72		

			0.546	12.76		
		100	0.422	34.14	31.95±1.30	
			0.434	32.07		
			0.448	29.66		
		500	0.307	53.97	54.02±1.05	
			0.317	52.24		
			0.296	55.86		
		1000	0.255	62.93	66.49±1.79	
			0.226	67.93		
			0.222	68.62		
		1500	0.196	73.10	78.45±3.62	
			0.174	76.90		
			0.125	85.34		
		2000	0.115	87.07	88.05±1.34	
			0.119	86.38		
			0.094	90.69		
Termitomyces schimperi	Methanol	10	0.542	13.45	6.49±3.66	0.953
			0.591	5.00		
			0.614	1.03		
		25	0.535	14.66	9.08±3.84	
			0.557	10.86		
			0.61	1.72		
		50	0.514	18.28	17.93±0.62	
			0.511	18.79		
			0.523	16.72		
		100	0.493	21.90	24.37±1.56	
			0.462	27.24		

			0.481	23.97		
		500	0.367	43.62	42.59±1.39	
			0.363	44.31		
			0.389	39.83		
		1000	0.242	65.17	59.77±2.79	
			0.282	58.28		
			0.296	55.86		
		1500	0.226	67.93	66.67±2.28	
			0.215	69.83		
			0.259	62.24		
		2000	0.14	82.76	84.43±1.50	
			0.138	83.10		
			0.113	87.41		

Appendix 2: DPPH scavenging activity of water extracts of mushroom samples

Species	Solvent	Conc (µg/mL)	Abs.	Inhi. %	Mean %	IC50 (mg/mL)
Agaricus bisporus	Water	10	0.625	2.50	2.67±0.10	1.401
			0.624	2.67		
			0.623	2.83		
		25	0.583	9.50	9.06±0.99	
			0.597	7.17		
			0.577	10.50		
		50	0.546	15.67	15.50±0.10	
			0.547	15.50		
			0.548	15.33		
		100	0.517	20.50	20.67±0.25	
			0.518	20.33		
			0.513	21.17		
		500	0.404	39.33	36.22±1.78	
			0.441	33.17		
			0.423	36.17		
		1000	0.404	39.33	38.89±0.24	
			0.407	38.83		
			0.409	38.50		
		1500	0.355	47.50	48.39±0.65	
			0.342	49.67		
			0.352	48.00		
		2000	0.246	65.67	66.61±1.66	
			0.221	69.83		
			0.254	64.33		

Lentinula edodes	Water	10	0.625	2.50	2.06±0.62	1.636
			0.623	2.83		
			0.635	0.83		
		25	0.599	6.83	6.72±1.06	
			0.611	4.83		
			0.589	8.50		
		50	0.554	14.33	13.56±0.43	
			0.559	13.50		
			0.563	12.83		
		100	0.545	15.83	17.33±0.75	
			0.532	18.00		
			0.531	18.17		
		500	0.453	31.17	31.72±0.47	
			0.452	31.33		
			0.444	32.67		
		1000	0.431	34.83	35.61±0.40	
			0.423	36.17		
			0.425	35.83		
		1500	0.368	45.33	46.50±0.73	
			0.362	46.33		
			0.353	47.83		
		2000	0.307	55.50	56.00±0.25	
			0.302	56.33		
			0.303	56.17		
Pleurotus ostreatus	Water	10	0.606	5.67	6.17±0.25	1.458
			0.602	6.33		
			0.601	6.50		

		25	0.548	15.33	16.94±0.84	
			0.536	17.33		
			0.531	18.17		
		50	0.542	16.33	16.33±0.19	
			0.544	16.00		
			0.54	16.67		
		100	0.551	14.83	16.50±0.86	
			0.534	17.67		
			0.538	17.00		
		500	0.542	16.33	17.61±0.83	
			0.536	17.33		
			0.525	19.17		
		1000	0.441	33.17	36.67±1.76	
			0.408	38.67		
			0.411	38.17		
		1500	0.305	55.83	55.67±0.44	
			0.302	56.33		
			0.311	54.83		
		2000	0.258	63.67	62.89±0.86	
			0.257	63.83		
			0.273	61.17		
Scleroderma cepa	Water	10	0.664	-4.00	2.78±3.39	0.935
			0.601	6.50		
			0.605	5.83		
		25	0.598	7.00	8.11±0.56	
			0.587	8.83		
			0.589	8.50		

		50	0.557	13.83	14.72±0.53	
			0.552	14.67		
			0.546	15.67		
		100	0.472	28.00	28.72±0.36	
			0.466	29.00		
			0.465	29.17		
		500	0.403	39.50	39.28±0.78	
			0.413	37.83		
			0.397	40.50		
		1000	0.248	65.33	65.33±3.27	
			0.282	59.67		
			0.214	71.00		
		1500	0.227	68.83	69.89±0.63	
			0.221	69.83		
			0.214	71.00		
		2000	0.148	82.00	83.39±0.73	
			0.133	84.50		
			0.138	83.67		
Termitomyces microcarpus	Water	10	0.594	7.67	7.22±0.24	0.920
			0.597	7.17		
			0.599	6.83		
		25	0.554	14.33	14.39±0.15	
			0.552	14.67		
			0.555	14.17		
		50	0.511	21.50	20.06±0.73	
			0.523	19.50		
			0.525	19.17		

		100	0.481	26.50	26.83±0.42	
			0.482	26.33		
			0.474	27.67		
		500	0.345	49.17	49.00±0.17	
			0.348	48.67		
			0.345	49.17		
		1000	0.304	56.00	56.94±0.49	
			0.294	57.67		
			0.297	57.17		
		1500	0.251	64.83	64.17±0.51	
			0.253	64.50		
			0.261	63.17		
		2000	0.124	86.00	86.22±0.40	
			0.126	85.67		
			0.118	87.00		
Termitomyces schimperi	Water	10	0.599	6.83	8.67±0.93	0.925
			0.581	9.83		
			0.584	9.33		
		25	0.547	15.50	16.22±0.98	
			0.531	18.17		
			0.55	15.00		
		50	0.521	19.83	18.61±0.78	
			0.527	18.83		
			0.537	17.17		
		100	0.456	30.67	30.83±1.11	
			0.466	29.00		
			0.443	32.83		

		500	0.328	52.00	51.22±0.78	
			0.342	49.67		
			0.328	52.00		
		1000	0.303	56.17	54.50±1.07	
			0.311	54.83		
			0.325	52.50		
		1500	0.228	68.67	67.50±1.42	
			0.225	69.17		
			0.252	64.67		
		2000	0.164	79.33	80.72±1.15	
			0.142	83.00		
			0.161	79.83		

Appendix 3: ABTS scavenging assay of methanol extract of mushroom species

Species	Solvent	Conc (µg/mL)	Abs.	Inhi. %	Mean %	IC50 (mg/mL)
Agaricus bisporus	Water	10	0.625	2.50	2.67±0.10	1.401
			0.624	2.67		
			0.623	2.83		
		25	0.583	9.50	9.06±0.99	
			0.597	7.17		
			0.577	10.50		
		50	0.546	15.67	15.50±0.10	
			0.547	15.50		
			0.548	15.33		
		100	0.517	20.50	20.67±0.25	
			0.518	20.33		
			0.513	21.17		
		500	0.404	39.33	36.22±1.78	
			0.441	33.17		
			0.423	36.17		
		1000	0.404	39.33	38.89±0.24	
			0.407	38.83		
			0.409	38.50		
		1500	0.355	47.50	48.39±0.65	
			0.342	49.67		
			0.352	48.00		
		2000	0.246	65.67	66.61±1.66	
			0.221	69.83		
			0.254	64.33		

Lentinula edodes	Water	10	0.625	2.50	2.06±0.62	1.636
			0.623	2.83		
			0.635	0.83		
		25	0.599	6.83	6.72±1.06	
			0.611	4.83		
			0.589	8.50		
		50	0.554	14.33	13.56±0.43	
			0.559	13.50		
			0.563	12.83		
		100	0.545	15.83	17.33±0.75	
			0.532	18.00		
			0.531	18.17		
		500	0.453	31.17	31.72±0.47	
			0.452	31.33		
			0.444	32.67		
		1000	0.431	34.83	35.61±0.40	
			0.423	36.17		
			0.425	35.83		
		1500	0.368	45.33	46.50±0.73	
			0.362	46.33		
			0.353	47.83		
		2000	0.307	55.50	56.00±0.25	
			0.302	56.33		
			0.303	56.17		
Pleurotus ostreatus	Water	10	0.606	5.67	6.17±0.25	1.458
			0.602	6.33		
			0.601	6.50		

		25	0.548	15.33	16.94±0.84	
			0.536	17.33		
			0.531	18.17		
		50	0.542	16.33	16.33±0.19	
			0.544	16.00		
			0.54	16.67		
		100	0.551	14.83	16.50±0.86	
			0.534	17.67		
			0.538	17.00		
		500	0.542	16.33	17.61±0.83	
			0.536	17.33		
			0.525	19.17		
		1000	0.441	33.17	36.67±1.76	
			0.408	38.67		
			0.411	38.17		
		1500	0.305	55.83	55.67±0.44	
			0.302	56.33		
			0.311	54.83		
		2000	0.258	63.67	62.89±0.86	
			0.257	63.83		
			0.273	61.17		
Scleroderma cepa	Water	10	0.664	-4.00	2.78±3.39	0.935
			0.601	6.50		
			0.605	5.83		
		25	0.598	7.00	8.11±0.56	
			0.587	8.83		
			0.589	8.50		

		50	0.557	13.83	14.72±0.53	
			0.552	14.67		
			0.546	15.67		
		100	0.472	28.00	28.72±0.36	
			0.466	29.00		
			0.465	29.17		
		500	0.403	39.50	39.28±0.78	
			0.413	37.83		
			0.397	40.50		
		1000	0.248	65.33	65.33±3.27	
			0.282	59.67		
			0.214	71.00		
		1500	0.227	68.83	69.89±0.63	
			0.221	69.83		
			0.214	71.00		
		2000	0.148	82.00	83.39±0.73	
			0.133	84.50		
			0.138	83.67		
Termitomyces microcarpus	Water	10	0.594	7.67	7.22±0.24	0.920
			0.597	7.17		
			0.599	6.83		
		25	0.554	14.33	14.39±0.15	
			0.552	14.67		
			0.555	14.17		
		50	0.511	21.50	20.06±0.73	
			0.523	19.50		
			0.525	19.17		

		100	0.481	26.50	26.83±0.42	
			0.482	26.33		
			0.474	27.67		
		500	0.345	49.17	49.00±0.17	
			0.348	48.67		
			0.345	49.17		
		1000	0.304	56.00	56.94±0.49	
			0.294	57.67		
			0.297	57.17		
		1500	0.251	64.83	64.17±0.51	
			0.253	64.50		
			0.261	63.17		
		2000	0.124	86.00	86.22±0.40	
			0.126	85.67		
			0.118	87.00		
Termitomyces schimperi	Water	10	0.599	6.83	8.67±0.93	0.925
			0.581	9.83		
			0.584	9.33		
		25	0.547	15.50	16.22±0.98	
			0.531	18.17		
			0.55	15.00		
		50	0.521	19.83	18.61±0.78	
			0.527	18.83		
			0.537	17.17		
		100	0.456	30.67	30.83±1.11	
			0.466	29.00		
			0.443	32.83		

		500	0.328	52.00	51.22±0.78	
			0.342	49.67		
			0.328	52.00		
		1000	0.303	56.17	54.50±1.07	
			0.311	54.83		
			0.325	52.50		
		1500	0.228	68.67	67.50±1.42	
			0.225	69.17		
			0.252	64.67		
		2000	0.164	79.33	80.72±1.15	
			0.142	83.00		
			0.161	79.83		

Appendix 4: ABTS scavenging assay of water extract of mushroom species

Species	Solvent	Conc.(μg/mL)	Abs.	Inhi. %	Mean %	IC50 (mg/mL)
Agaricus bisporus	Water	10	0.625	0.86	3.22±2.02	1.053
			0.621	1.55		
			0.588	7.24		
		25	0.591	6.72	11.32±4.34	
			0.588	7.24		
			0.514	20.00		
		50	0.528	17.59	15.23±2.71	
			0.524	18.28		
			0.573	9.83		
		100	0.494	23.45	26.15±1.39	
			0.467	28.10		
			0.474	26.90		
		500	0.406	38.62	34.94±1.93	
			0.444	32.07		
			0.432	34.14		
		1000	0.351	48.10	50.23±1.06	
			0.332	51.38		
			0.333	51.21		
		1500	0.269	62.24	67.36±2.84	
			0.212	72.07		
			0.237	67.76		
		2000	0.164	80.34	78.91±1.04	
			0.184	76.90		
			0.169	79.48		

Lentinula edodes	Water	10	0.582	8.28	4.83±2.42	0.991
			0.629	0.17		
			0.595	6.03		
		25	0.576	9.31	7.47±1.36	
			0.602	4.83		
			0.582	8.28		
		50	0.562	11.72	11.67±2.64	
			0.589	7.07		
			0.536	16.21		
		100	0.569	10.52	16.21±2.87	
			0.523	18.45		
			0.516	19.66		
		500	0.454	30.34	28.45±2.43	
			0.493	23.62		
			0.448	31.38		
		1000	0.247	66.03	61.49±4.12	
			0.321	53.28		
			0.252	65.17		
		1500	0.201	73.97	72.70±0.64	
			0.213	71.90		
			0.211	72.24		
		2000	0.195	75.00	84.77±4.89	
			0.109	89.83		
			0.111	89.48		
Pleurotus ostreatus	Water	10	0.566	11.03	10.98±0.75	1.364
			0.574	9.66		
			0.559	12.24		

		25	0.523	18.45	17.76±1.34	
			0.542	15.17		
			0.516	19.66		
		50	0.508	21.03	21.15±0.40	
			0.511	20.52		
			0.503	21.90		
		100	0.475	26.72	24.37±1.19	
			0.497	22.93		
			0.494	23.45		
		500	0.478	26.21	29.08±1.56	
			0.447	31.55		
			0.459	29.48		
		1000	0.412	37.59	36.15±1.52	
			0.438	33.10		
			0.411	37.76		
		1500	0.321	53.28	53.28±0.60	
			0.327	52.24		
			0.315	54.31		
		2000	0.246	66.21	67.64±0.85	
			0.238	67.59		
			0.229	69.14		
Scleroderma cepa	Water	10	0.545	14.66	13.91±0.45	0.583
			0.549	13.97		
			0.554	13.10		
		25	0.518	19.31	22.07±1.64	
			0.503	21.90		
			0.485	25.00		

		50	0.478	26.21	27.07±2.48	
			0.446	31.72		
			0.495	23.28		
		100	0.474	26.90	31.09±2.60	
			0.453	30.52		
			0.422	35.86		
		500	0.278	60.69	61.09±0.68	
			0.268	62.41		
			0.281	60.17		
		1000	0.163	80.52	78.33±3.09	
			0.153	82.24		
			0.211	72.24		
		1500	0.091	92.93	91.49±0.97	
			0.11	89.66		
			0.097	91.90		
		2000	0.085	93.97	96.09±1.42	
			0.076	95.52		
			0.057	98.79		
Termitomyces microcarpus	Water	10	0.565	11.21	10.34±1.60	0.630
			0.588	7.24		
			0.557	12.59		
		25	0.546	14.48	20.86±3.39	
			0.479	26.03		
			0.502	22.07		
		50	0.435	33.62	29.94±2.64	
			0.486	24.83		
			0.448	31.38		

		100	0.398	40.00	37.70±1.34	
			0.411	37.76		
			0.425	35.34		
		500	0.311	55.00	55.23±1.20	
			0.297	57.41		
			0.321	53.28		
		1000	0.207	72.93	69.89±1.62	
			0.239	67.41		
			0.228	69.31		
		1500	0.129	86.38	88.79±1.21	
			0.109	89.83		
			0.107	90.17		
		2000	0.09	93.10	92.82±0.57	
			0.087	93.62		
			0.098	91.72		
Termitomyces shimperi	Water	10	0.598	5.52	3.45±1.05	1.226
			0.618	2.07		
			0.614	2.76		
		25	0.602	4.83	5.11±0.86	
			0.591	6.72		
			0.608	3.79		
		50	0.573	9.83	7.13±1.39	
			0.6	5.17		
			0.593	6.38		
		100	0.566	11.03	11.67±0.32	
			0.561	11.90		
			0.56	12.07		

		500	0.431	34.31	31.32±2.41	
			0.476	26.55		
			0.438	33.10		
		1000	0.311	55.00	51.32±1.88	
			0.339	50.17		
			0.347	48.79		
		1500	0.298	57.24	58.79±0.90	
			0.28	60.34		
			0.289	58.79		
		2000	0.22	70.69	67.64±1.79	
			0.256	64.48		
			0.237	67.76		

PHOTOPLATES

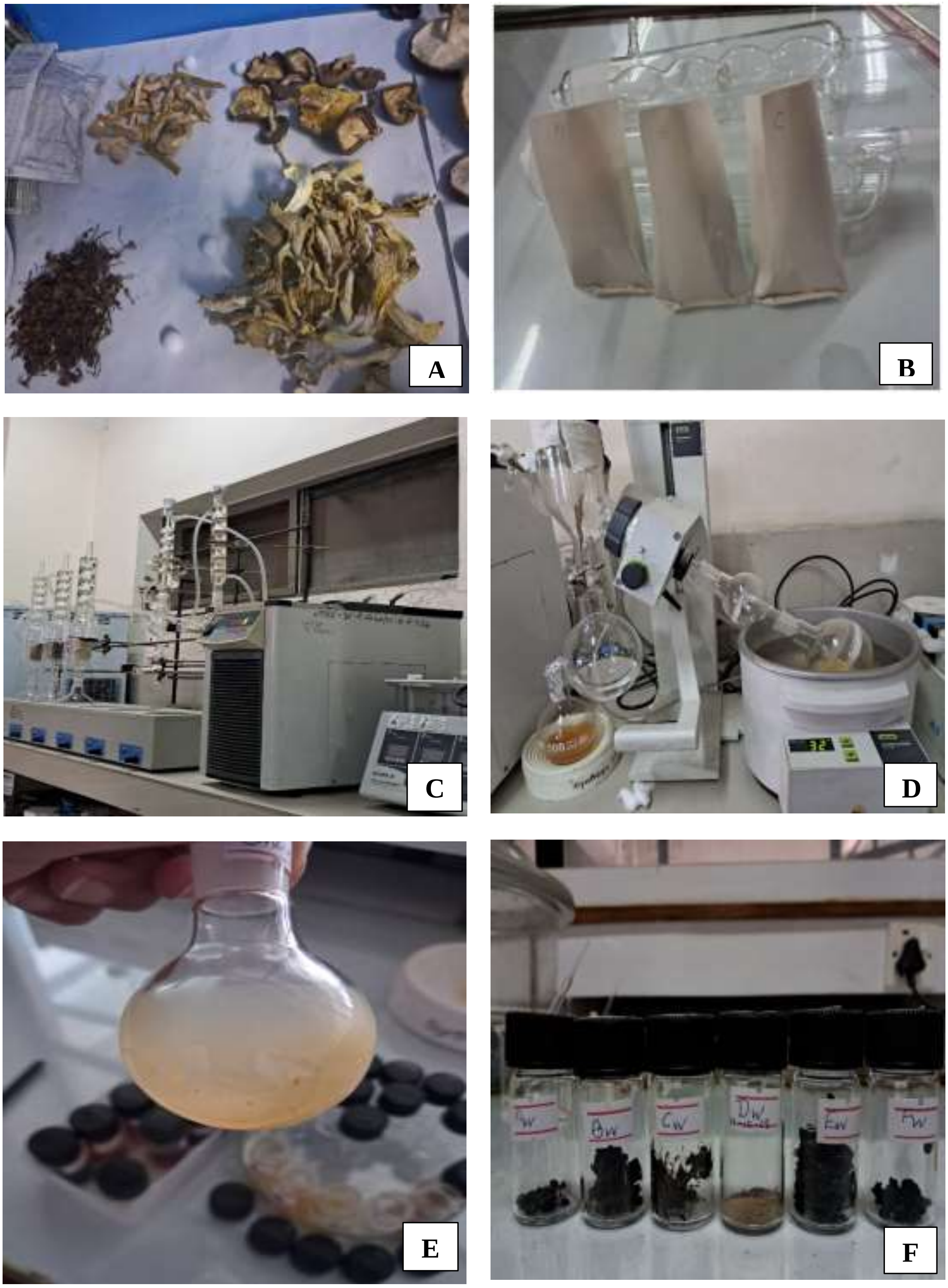

Photo plate 1: A. Dried mushroom sample. B. Thimbles. C. Soxhlet extraction. D. Rotary evaporation. E. Extracted sample. F. Dried extracts

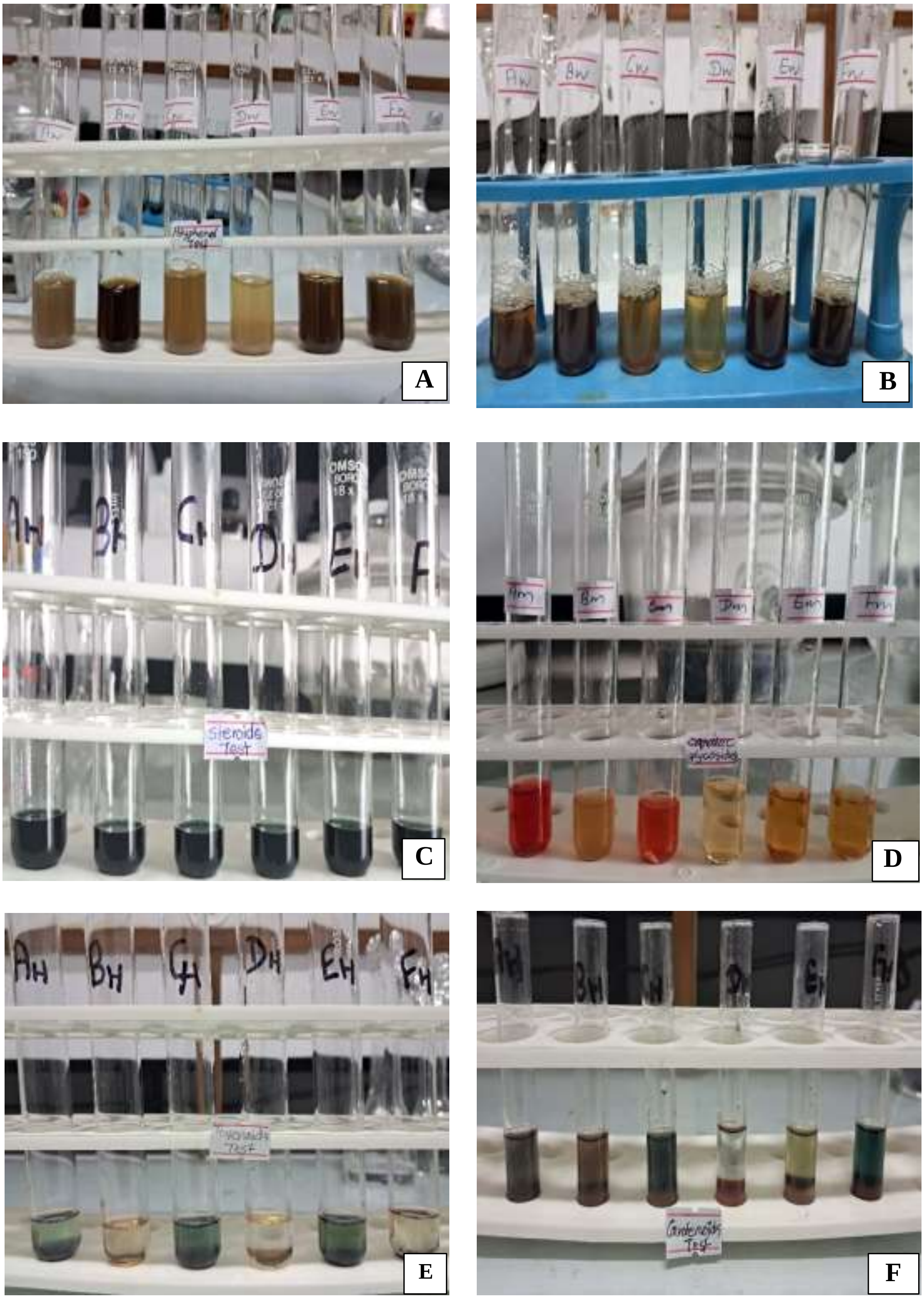

Photo plate 2: A. Polyphenol test. B. Saponin test. C. Steroid test. D. Cardiac glycosides test. E. Terpenoid test. F. Carotenoids test

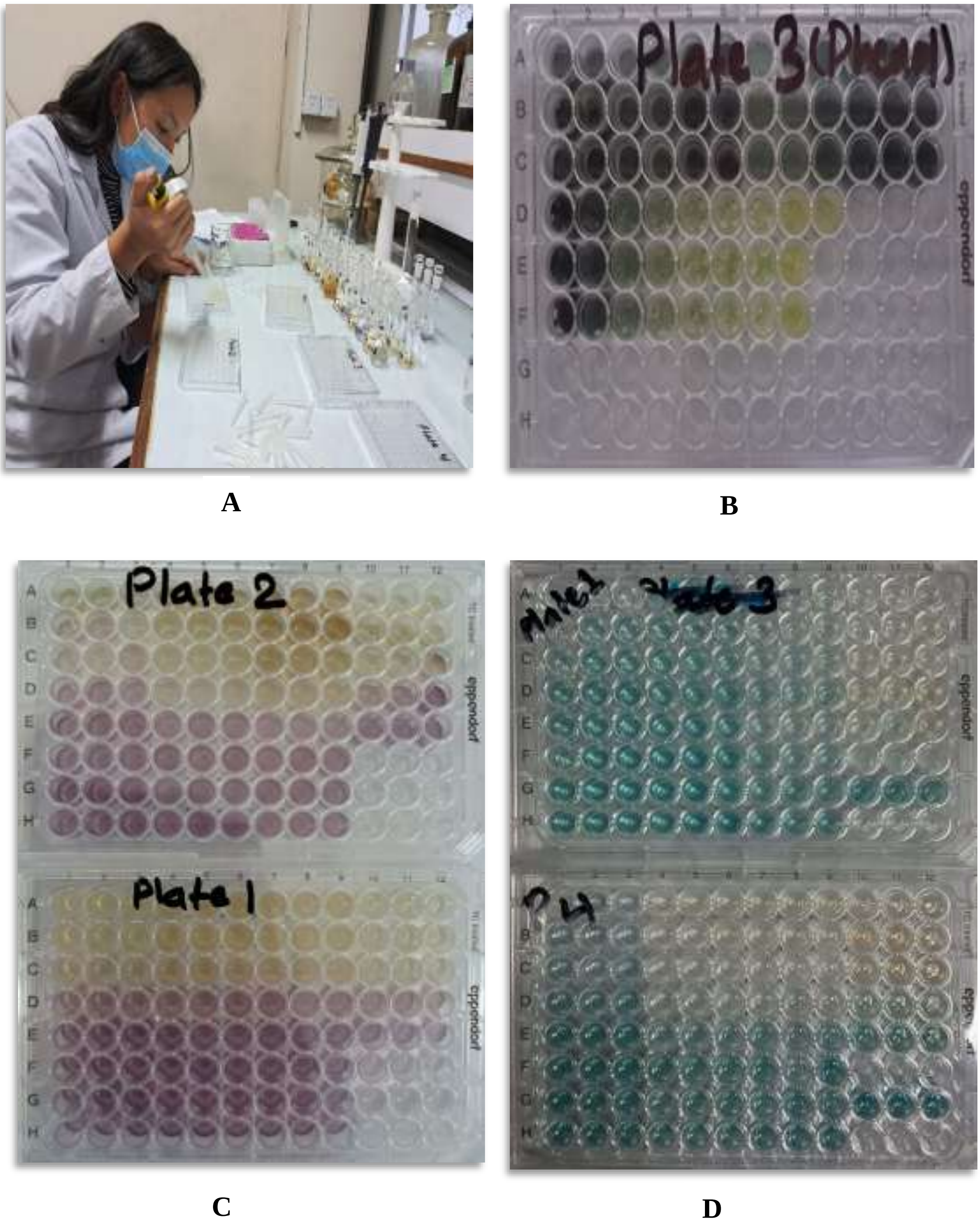

Photo plate 3: A. Testing antioxidant activity. B. Phenolic determination. C. DPPH assay. D. ABTS assay